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Effect of niacin and its analogs on growth and on the glucose catabolic pathways of *Staphylococcus aureus*

by

Manohar V. Wadke

A Dissertation submitted to the faculty of the graduate school of Loyola University of Chicago, in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Effect of niacin and its analogs on growth and on the glucose catabolic pathways of *Staphylococcus aureus*.

Monohar WADKE
Loyola University of Chicago Stritch School of Medicine, Maywood, Illinois
Advisor: Dr. H. J. Blumenthal

S. aureus catabolized glucose by both the Embden-Meyerhof (EM) and the hexosemonophosphate (HMP) pathways, as determined with ^{14}C-labeled glucose. The relative activities of these pathways, and of the TCA cycle, were influenced by the inclusion of niacin and/or thiamine in the growth medium. When > 0.25 μg/ml of niacin was added to 2% Vitamin-free Casitone, the proportion of glucose metabolized via the HMP pathway by *S. aureus* was stimulated 3-fold. When 0.1 μg/ml of niacin was added, the growth was maximally stimulated but increased NAD(P) synthesis or HMP pathway activity was not observed. Under growing conditions, increased glucose oxidation via the HMP pathway and TCA cycle activity were correlated with the increased synthesis of NADP-specific glucose-6-P dehydrogenase and isocitrate dehydrogenase, respectively. When the vitamin supplementation was made to resting niacin deficient cells, metabolic changes similar to those in growing cells occurred, even in the presence of protein inhibitors such as chloramphenicol, puromycin or actinomycin D. Since the specific activities of the enzymes tested remained unchanged under conditions that precluded new enzyme synthesis, the enzyme levels were not the limiting factors for glucose oxidation via the HMP pathway.

In order to determine if either NAD and/or NADP were regulating the HMP pathway activity, the staphylococcal suspensions were starved for 6 hr in buffered-glucose (pH 7.0), under conditions that allowed the NAD to decrease and NADP to remain constant. During the period of starvation the initial NAD level decreased to 1 μmole/g and the percent of glucose oxidized via the HMP pathway decreased to 9%, reaching a value of 6% after 6 hr at which time the NAD content was further reduced to 0.5 μmole/g. When niacin was added, the NAD levels and the HMP activity in these cells were increased to 1.2 μmoles and 16%, respectively, after 2 hr of additional incubation. These results indicate that NAD is regulating the activity of this expandable (6 to 22%) HMP pathway, by increasing NADP turnover via the transhydrogenase system.

Any of several 1-,2-,3- and 4-substituted niacin analogs completely replaced the niacin requirement for both growth and the HMP pathway utilization when added at high concentration (ca. 1000 μg/ml) to either a synthetic medium or Vitamin-free Casitone supplemented with thiamine. On the basis of their effects on growth and on the HMP pathway the analogs were classified into 4 groups. The 22 goup I analogs, including pyridine-N-oxide, pyridine-3-sulfonic acid and 3-acetylpyridine, stimulated both growth and the HMP pathway maximally while the 9 group II analogs, such as picolinic acid, stimulated growth and the HMP pathway to a lesser extent. There were 13 analogs from group III, including 6-hydroxynicotinic acid, that were without effect while 4 analogs from group IV, including 6-aminonicotinamide, were inhibitory. Parallel experiments in this laboratory using labeled quinolinic acid, isoniazid and pyridine-N-oxide indicated that the analogs were converted to NAD(P) and not to their analogs. On the basis of the permeability studies with the labeled compounds and ^{14}C-niacin, it was suggested that the need for high concentrations of analogs for growth was due to the absence of active transport for these compounds and the analogs entered the cells by simple passive diffusion to cause biochemical changes similar to those caused by niacin.

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ABBREVIATIONS

3-AP 3-Acetylpyridine
AcPyAD .... 3-Acetylpyridine adenine dinucleotide
AMP .... Adenosine monophosphate
6-AN 6-Aminonicotinamide
ATP .... Adenosine triphosphate
DPT .... Diphosphothiamine
ED .... Entner-Doudoroff (pathway)
EM .... Embden-Meyerhof (pathway)
G6P Glucose-6-phosphate
G6PD .... Glucose-6-phosphate dehydrogenase
GTP .... Guanosine triphosphate
HMP .... Hexosemonophosphate (pathway)
6-HN .... 6-Hydroxynicotinic acid
ICD .... Isocitrate dehydrogenase
NAD .... Nicotinamide adenine dinucleotide
NADH .... Nicotinamide adenine dinucleotide-reduced
NADP .... Nicotinamide adenine dinucleotide phosphate
NADPH .... Nicotinamide adenine dinucleotide phosphate-reduced
NDA .... Nicotinyldiethyl amide
NNO .... Nicotinamide-N-oxide
OD .... Optical density
PC 3-Pyridycarbinol
6PG 6-Phosphogluconate
6PGD .... 6-Phosphogluconate dehydrogenase
PSA .... Pyridine-3-sulfonic acid
TCA .... Tricarboxylic acid cycle
TH .... Transhydrogenase
ThN .... Thionicotinamide
VFC .... Vitamin-free Casitone

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DEDICATION

To my wife, Nenita, whose sacrifice, guidance and encouragement have brought meaning and direction to my life.

INTRODUCTION

Glucose is the main metabolic fuel for living cells, providing both energy and intermediates for various biosynthetic reactions. Although important insights have already been obtained during the past decade into the regulatory mechanisms which influence microbial activities such as amino acid biosynthesis, the same degree of knowledge is not available for the regulation of carbohydrate metabolism. In bacteria the control mechanisms for individual glycolytic pathways, particularly the Embden-Meyerhof (EM) pathway, are known to a moderate degree. For example, when cells catabolize glucose via the EM pathway, phosphofructokinase plays an important regulatory role on this activity. However, when the cell is synthesizing glucose from smaller molecules through a reversal of the EM pathway, then the enzyme fructose-1,6-diphosphatase plays the important role of controlling the activity of this pathway. The situation is more complex when we wish to learn about the factors that control the amount of glucose being utilized by the two pathways that can both utilize glucose within the cells, the EM and the hexosemonophosphate (HMP) pathways. By comparison, very little is known about such regulatory factors.

Although the importance of the vitamins niacin and thiamine in microbial glycolysis is known, their effects on the individual glycolytic pathways are relatively unexplored. Regulation of the HMP pathway by the availability of NADP and by the levels

of some glycolytic enzymes has been demonstrated in various bacteria. However, NAD has not been shown to be involved in the control reactions of the pentose phosphate pathway. The lack of information on the role of NAD from a regulatory point of view mainly stems from the fact that NAD does not generally serve as a coenzyme in the HMP pathway.

The present study is an extension of an earlier study which had shown that the addition of niacin resulted in a 3- to 4-fold stimulation in utilization of the HMP pathway of S. aureus and a concomitant decrease in the percentage of glucose metabolized via the EM pathway. In order to investigate some of the factors involved in the regulation of glucose oxidation via the HMP pathway, the levels of glycolytic enzymes and of nicotinamide coenzymes were studied in cells under conditions in which the HMP pathway was increased or decreased. Also, niacin analogs were employed in these studies in an attempt to obtain more information about the regulatory role of NAD(P). Since niacin analogs were found to completely replace niacin, some studies on the mechanism of niacin replacement by the analogs were also initiated.

I. REVIEW OF LITERATURE

Glycolysis usually serves to satisfy various cellular requirements such as energy and precursors for biosynthesis. In addition glycolysis serves to convert these precursors into useful intermediates for the appropriate cellular reactions. Staphylococcus aureus metabolizes glucose aerobically or anaerobically by both the Embden-Meyerhof (EM) and the hexosemonophosphate (HMP) pathways (88, 113), with the EM being the major pathway. Under aerobic conditions the pyruvate is further oxidized to water and CO_2 via the tricarboxylic acid (TCA) cycle (94,111). So far there is only negative evidence in the literature for the occurence of the Entner-Doudoroff (ED) pathway in staphylococci (113).

The HMP pathway (also known as the pentose phosphate pathway or the pentose cycle) for glucose catabolism is widely distributed in microorganisms. The pathway provides a route of glucose oxidation that is independent of the TCA cycle and of glycolysis by the EM pathway. It is a generator of NADPH and of 4 and 7 carbon sugars necessary for various biosynthetic processes. It is also a source of ribose, an intermediate product of this pathway that can be used for synthesis of nucleic acid.

Operation of the HMP pathway along with the EM pathway during glycolysis became apparent in a wide variety of living

cells. During the investigation of biochemical changes in *S. aureus*, some evidence indicating the operation of the HMP pathway was obtained, by measuring the rate of formation of $^{14}CO_2$ from glucose-1-^{14}C and glucose-6-^{14}C (19, 38).

Oxidation of glucose has been studied in *S. aureus* in the presence or absence of arsenite, an inhibitor which causes the accumulation of pyruvate and thus blocks the further oxidation of pyruvate via the TCA cycle (113). In the presence of this metabolic inhibitor, considerable amounts of $^{14}CO_2$ were recovered during the oxidation of glucose-1-^{14}C and glucose-U-^{14}C. Thus the results of these studies demonstrated the simultaneous operation of the HMP pathway along with the EM pathway and established the importance of the HMP pathway in staphylococcal carbohydrate metabolism.

Estimation of the glucose catabolic pathways also indicated that about 64% of the glucose was oxidized via the HMP pathway by resting cell suspension previously grown in a broth lacking glucose . When glucose was added to the nutrient broth, glucose oxidation through the HMP pathway decreased from 64% to 34%. In addition there was an inhibition of the TCA cycle. Studies on various individual glycolytic enzymes were also made and evidence for repression (i.e., inhibition or suppression of the synthesis of enzymes) was obtained when glucose was present in the medium (16,49,

113). This effect of glucose has also been reported for other microorganisms, such as Bacillus subtilis, Escherichia coli and Thiobacillus intermedius (36,39).

Glucose repression of cellular activities is not limited to microbes. For instance, glucose inhibited the glucose oxidation via the TCA cycle in ascites tumor cells; however, it increased the oxidation of carbon-1 of glucose, presumably by the HMP pathway (95). At the same time it was also demonstrated in S. aureus that addition of glucose to the growth medium reduced both the oxidation of glucose by the HMP pathway and the subsequent oxidation of pyruvate via the TCA cycle.

The iron content of the medium also has a significant effect on the glycolytic pathways in staphylococci (116). The metabolic activites of S. aureus grown in an iron-poor and in an iron-rich Trypticase medium, with and without addition of glucose,were compared. It was found that in the absence of glucose, iron-rich cells oxidized glucose and other substrates more efficiently than did the cells grown in the iron restricted medium. The addition of glucose under the same conditions resulted in severe reduction of the oxidative capacity of the iron-restricted cells, compared to that of iron-rich cells. From these results it was concluded that the metabolic activity of S. aureus was affected by

both the amount of iron in the medium and by the presence or absence of glucose.

The effects of a number of different physical and physiological conditions that might alter the glycolytic pathways in *S. aureus* have been studied by various workers and reviewed by Blumenthal (5). The effect of vitamins on the glycolytic pathways have not been extensively investigated in microorganisms. A thiamine deficiency in red blood cells is known to diminish the activity of the HMP pathway since DPT, the coenzyme form of thiamine, is a coenzyme for the HMP pathway enzyme transketolase. It has been shown that the addition of thiamine to thiamine-deficient red blood cells stimulated the activity of transketolase which in turn stimulated the HMP pathway (102).

Earlier studies with staphylococci yielded information of a general nature. A certain relationship between niacin and thiamine and the utilization of glucose by staphylococci was observed (61). In order to elucidate the specific roles of these two vitamins in glucose metabolism by this organism, staphylococci were grown in 10% Vitamin-free casein hydrolysate containing constant amounts of niacin and thiamine (1.0 µg/ml). It was demonstrated that there was an active aerobic growth of staphylococci in the presence of niacin alone, with only partial utilization of glucose. The amount

of glucose metabolized was only about 40% of the amount used in the presence of niacin and thiamine. Analysis of the end products of glucose catabolism also indicated that thiamine catalyzed the oxidation of pyruvate only in the presence of niacin; however, specific effects of these two vitamins on the glucose catabolic pathways were not investigated.

The formation of niacin coenzymes, NAD and NADP, from niacin with the subsequent stimulation of glycolysis in S. aureus has been noted (47). During the investigation on the effects of some niacin analogs on glycolysis, marked glycolytic inhibition as a result of the addition of niacin analogs was noted. Based on these results it was suggested that the analogs probably acted by interferring with the staphylococcal synthesis of nicotinamide coenzymes. Montiel and Blumenthal (81), using labelled glucose to estimate the pathways in living cells, showed that the addition of niacin and thiamine to the growth medium had significant effects on the degree of operation of the EM and the HMP pathways. They reported that the addition of niacin to the growth medium increased the operation of the HMP pathway, while both niacin and thiamine were necessary for maximal stimulation of the TCA cycle activity. The effect of niacin and thiamine on the coenzyme levels was investigated (44). It was shown that when niacin was added to the medium, there was a 15- to 30-fold increase in NAD level and only a

2-fold increase in NADP, NADH and NADPH levels in staphylococci. This increase in the coenzyme contents due to the vitamins was also followed by stimulation of glycolysis in *S. aureus* (45).

Participation of glucose catabolic pathways in microorganisms. Wang et al. (119) introduced the radiorespirometric method for the estimation of the glucose catabolic pathways. This method has been widely and successfully used to evaluate the glycolytic pathways in many different bacteria. Based on this technique it was reported that in *Pseudomonas natrigens* 92% of the glucose was catabolized via the EM pathway, whereas 8% was oxidized via the HMP pathway. The relative participation of the EM and the HMP pathways in *Escherichia coli* was 72% and 28%, respectively (23). On the other hand, complete absence of the EM and the ED pathways was noted in *Acetobacter suboxydans*, with all of the glucose catabolized via the HMP pathway (60). Similarly, the EM and the HMP pathways were shown not to be operative in *Pseudomonas saccharophila* (12). When *S. aureus* was grown in Trypticase soy broth (which contains glucose), the simultaneous participation of the EM and the HMP pathways was 88.7% and 11.3%, respectively (4). Montiel and Blumenthal (81) studied the effects of vitamins on glucose catabolic pathways in various strains of *S. aureus* and *S. epidermidis*. Their findings demonstrated that in the presence of niacin and thiamine

roughly 14-40% of the glucose was oxidized via the HMP pathway by staphylococci.

Generally, in purely biosynthetic pathways the major control exerted is the negative feedback control. In this type of control process, the end product or the metabolite represses the synthesis and/or inhibits the activity of the first enzyme of that pathway. In catabolic pathways, however, the activity of initiating enzyme is generally controlled by compounds which serve as indicators of the energy state of a cell such as inorganic phosphate, pyrophosphate, purine or pyrimidine nucleotides. Occurance of cooperative feedback, concerted feedback, cumulative feedback and precursor activation have been studied in detail (84, 90, 127).

The various control mechanisms involved in amphibolic pathways (i.e., metabolic pathway providing both anabolic and catabolic functions) have been recently reviewed by Sanwal (101). There are various diverse control mechanisms present which differ in details from microbe to microbe, although the main types of control mechanisms are the same. This is in agreement with the concept of "unity of biochemistry". For example, citrate synthase in animal (50), in Yeast(89) or in *B. subtilis* (28) is inhibited by ATP, while in *E. coli* it is inhibited by NADH and α-ketoglutarate. In the case of aerobic Gram-positive bacteria such as staphylococci, the enzyme is not regulated by

either ATP or NADH, and probably some other different control mechanism may be present in these organisms. Moreover, many of the control mechanisms have been studied extensively only in *E. coli* and in few other Gram-negative bacilli. More detailed studies are needed in other groups of bacteria before a generalization can be made about the regulatory process. Recently it was reported that fructose-6-phosphate, glucose-6-phosphate and AMP inhibited the activity of two enzymes in the aspartic acid family of *E. coli* (2). This example of the control of amino acid biosynthesis by glycolytic intermediates of the EM and the HMP pathways again indicates the extent and importance of this type of control mechanism.

There is considerable evidence indicating that the activity of the hexosemonophosphate pathway may be partly controlled by the supply of NADP. The presence of artificial electron acceptors (9) or the supply of substrates which require NADPH for their metabolism (42, 57) can increase the rate of formation of $^{14}CO_2$ from glucose-1-^{14}C, suggesting active operation of the HMP pathway. Similar effects have been observed in other animal tissues such as erythrocytes (8), corneal epithelium (59) and ascites tumor cells (121). The HMP pathway in erythrocytes was shown to be dependent on the availability of NADP (18). Later Smith and Stourman, (110) using a hemolysate preparation of red blood cells, confirmed the enhancement of the HMP pathway activity following the addition of NADP to the extract. High

activities of glucose-6-phosphate and 6-phosphogluconate dehydrogenases together with the marked increase which occurred in the oxidation of carbon-1 of glucose when artificial electron acceptors were added to the medium indicated that the activity of the HMP pathway was limited by the supply of NADP (79).

The operation of the HMP pathway in _P. natrigens_ was rate limited by the supply of NADP (24). This was based on further studies on the rate of oxidation of NADH and NADPH and the activity of transhydrogenase in crude extracts from a number of organisms in which estimates of the relative degree of operation of the EM and the HMP pathways had already been made. A strong correlation was found between those microbes whose extracts possessed an active NADPH oxidase and transhydrogenase and those organisms which predominantly oxidized glucose through the HMP and/or ED pathways. The activities of these enzymes were absent in the organisms mainly utilizing glucose by the EM pathway. Rate limiting effects of NADP might also be overcome by the presence of two initial enzymes of HMP pathway that could utilize either NAD or NADP as cofactors (13). Such enzymes are extremely rare.

Thus the activities of the glycolytic pathways can be controlled by the availability of cofactors as well as by the levels of adenine nucleotides. In addition to this control by the nucleotides, the regulation of these pathways can be

influenced by the variation in the amounts of certain key enzymes of the pathway. Osmond and ApRees (87) suggested that in yeast, control of the HMP pathway occurred by variation in the amount of some enzymes. They tested the activities of certain enzymes of both EM and the HMP pathways in extracts of yeast grown either in simple medium containing nitrate as the sole nitrogen source, or in a complex medium containing all of the amino acids. Measurement of the enzyme activities was also correlated with the estimation of $^{14}CO_2$ production from -1-^{14}C and -6-^{14}C-glucose. Their findings demonstrated that the activities of G6PD and transketolase were significantly higher in extracts of yeast grown on the simple nitrate medium as compared to those grown on the complex amino acid medium. Increased enzyme activities paralleled increased $^{14}CO_2$ liberation from glucose-1-^{14}C, providing a rough measurement of the HMP pathway activity. Similarly, yeast cells were first grown in the complex medium and then transferred to the simple medium; this also resulted in the increased synthesis of the same enzymes of the HMP pathway. On the basis of these results they suggested that the increased amount of G6PD and transketolase indicated that adaptation to carbohydrate oxidation via the HMP pathway involved synthesis of more enzymes.

Regulation of the glycolytic pathways also depends on the induction, activation or inhibition of the glycolytic enzymes by vitamins, metabolites or coenzymes. For instance, induction

of G6PD synthesis by NADP, NADPH and by other compounds, such as nitrobenzimidazole, has been observed in animal and microbial cells (3, 105), indicating their role in the glycolytic control mechanisms. Thiamine, in the form of diphosphothiamine (DPT), has been shown to induce the synthesis of pyruvate decarboxylase in yeast, with a resultant increase in the rate of glycolysis (123). DPT is actually the coenzyme for pyruvate decarboxylase apoenzyme. This finding was based on the observation that pyruvate decarboxylase activity increased by 50% during the incubation of yeast cells with glucose and thiamine and by 300% when the cells were incubated with glucose, thiamine and nitrogen source. Similarly, this increased synthesis of the enzyme, induced by thiamine, was prevented by the inclusion of proflavin, p-fluorophenylalanine or 5-fluorouracil in the incubation medium. These results indicated that the reason for the higher activity of pyruvate decarboxylase, after incubation with thiamine, was a result of the stimulation of the *de novo* synthesis of the enzyme and not of an activation of preexisting enzyme molecules. There are also other examples of this type in animal tissues (37).

Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the oxidation of glucose-6-phosphate and thus introduces hexose into the pentose cycle. Since this is an important branching step in hexose metabolism, it is likely that this enzyme would be subjected to regulatory control. The G6PD from *Pseudomonas*

aeruginosa was strongly inhibited by ATP, GTP and dGTP by decreasing the binding of the substrate to the enzyme and it was suggested that adenine nucleotide control of the activity of G6PD might regulate the overall degradation of glucose under conditions requiring gluconeogenesis (70). A similar inhibition of G6PD of E. coli by ATP was noted, indicating that adenine nucleotide control of activity of allosteric enzymes may be of general importance (1). Recently it was reported that the activity of the G6PD of E. coli was inhibited in an allosteric manner by NADH. Although this inhibition of G6PD by NADH has not been observed in other bacterial species tested, it does appear to play a significant role in controlling the enzyme activity in E. coli (100).

If a cell has excess of ATP, it would appear logical to assume that it would have a feedback mechanism to slow down the glucose catabolism that is leading to the generation of more ATP. It was demonstrated that ATP and GTP were competitive inhibitors of G6PD, the important HMP allosteric enzyme in ascites tumor cells, while only 6PGD was sensitive to GTP (7). Schindler and Schlegel (103) concluded from their studies of G6PD from various microorganisms that G6PD functioned only in catabolism and that these enzymes were subjected to adenylate control with ATP as a negative allosteric effector. It is generally believed that phosphofructokinase is an important regulatory enzyme for the activity of the EM pathway and this it also is inhibited by

excess ATP and activated by AMP (117). If ATP is capable of inhibiting the key HMP pathway and the key EM pathway enzymes, high cellular levels of ATP should depress the total amount of glucose catabolism in the cell. This provides a sensitive and efficient mechanism for the regulation of the metabolic pathways (72, 93).

There is very little evidence regarding the control of other enzymes in the HMP pathway. The activity of transketolase was found to be limiting the HMP pathway activity in thiamine-deficient red blood cells (102). In addition, it has been reported that transketolase was the rate-limiting enzyme in the HMP pathway in most of the mammalian tissues studied (6, 85). In yeast, variation in transketolase activity was observed to parallel the degree of operation of the HMP pathway (87). Significance of this increased activity of transketolase may be that it prevented the accumulation of erythrose-4-phosphate, an inhibitor of glucose-phosphate isomerase, and allowed increased activity of the HMP pathway without any inhibition of the overall glycolytic rate.

The tricarboxylic acid cycle (TCA) functions mainly as an energy yielding process and also provides many precursors for cell biosynthesis and growth. The intermediates and biochemical reactions of this cycle have been extensively studied in numerous biological systems (67). Since many biosynthetic pathways

originate from the TCA cycle and many reactions of the cycle are important for the production of energy, it is likely that many of the enzymatic steps of this pathway are subjected to regulation by various effectors. One of the allosteric enzymes of this pathway has been found to be isocitrate dehydrogenase (ICD). This allosteric enzyme can thus increase or decrease the activity of the TCA cycle (75, 98, 99).

The control mechanisms for the activation of ICD are not fully understood. It has been demonstrated in *Neurospora*, and also in yeast (98) that the affinity of the substrate for the enzyme increases dramatically in the presence of AMP. This increased affinity may have significance in the metabolic regulation of the TCA cycle.

It has been suggested that the tricarboxylic acid cycle may be rate limited by the supply of NADP, since isocitrate dehydrogenase was shown to require NADP as a cofactor (14). In microorganisms two types of isocitrate dehydrogenases have been reported (101). NAD-specific isocitrate dehydrogenases are part of the normal function of the TCA cycle in the oxidation of substrate for the generation of energy; whereas NADP-specific isocitrate dehydrogenases are coupled to biosynthetic pathways originating from TCA cycle (34, 99).

Both niacin and thiamine have been considered to be essential vitamins for the growth of *S. aureus* for over 20 years.

In fact, it was with S. aureus that niacin was first proven to be a vitamin requirement for living cells (63). Although niacin remains as an absolute vitamin requirement for the growth of S. aureus, thiamine is no longer considered as an essential vitamin. Idriss and Blumenthal (48) developed a new thiamine-free synthetic medium for S. aureus which supported a moderate growth of staphylococci (OD, 0.2 in 10 hr) in the complete absence of thiamine when pyruvate or glucose was the carbon source. However, added thiamine still stimulated growth in an otherwise complete medium. Thus the inclusion of thiamine in the medium tripled the amount of growth.

Following the initial discovery and identification of these vitamins, a survey was made of the ability of several compounds closely related to niacin and thiamine to replace them as a growth factor for the growth of various bacteria (62, 63, 68, 69). The results of the studies showed that only a very limited departure in structure of the two vitamins could support the growth of staphylococci. S. aureus was the original microbe that was shown to require preformed nicotinic acid for growth. The utilization of a number of compounds related to nicotinic acid by various bacteria (i.e., S. aureus, Proteus vulgaris, Shigella dysenteriae and Lactobacillus plantarum) was studied by a number of workers (26, 27, 62, 73, 91); the results of these investigations have been well summarized by Koser (66).

The utilization of a number of isomers of nicotinic acid by *S. aureus* was studied, using amino acid-glucose medium (27, 69). The analogs were tested in the presence of thiamine (0.005 μg/ml) in the medium. Using concentrations of niacin isomers up to 2.0 μg/ml, it was reported that with the exception of nicotinuric acid, none of the following compounds could replace niacin in supporting growth of *S. aureus*: nicotinyldiethylamide, quinolinic acid, 2-methylpyridine, or isonicotinic acid. On the basis of these results it was concluded that the ability of *S. aureus* to utilize compounds related to niacin was very limited (68, 69).

Knight and McIlwain (63) investigated the ability of a series of pyridine derivatives to replace niacin, by adding them to a basal medium which was nutrionally adequate for the growth of *S. aureus*, except for niacin. All the analogs were tested at a concentration of 10^{-4}M in the presence of excess thiamine (10^{-7}M); the growth was measured for a period of 3 days under aerobic conditions. They reported that quinolinic acid, picolinic acid, isonicotinic acid, pyridine-3-sulfonic acid, 3-picoline and several other related pyridine derivatives could not replace niacin in supporting growth of *S. aureus*. On the basis of these results they suggested that niacin and thiamine were highly specific in their growth requirement for *S. aureus*.

Pyridine-3-sulfonic acid (PSA), the sulfur analog of niacin, was ineffective in replacing the niacin requirement for the growth of *Proteus* species. However, it strongly inhibited the growth of *S. aureus*, presumably by acting as an antimetabolite. The effect of various concentrations of PSA on the growth of *S. aureus* in the synthetic medium containing niacin and thiamine was studied (78). It was found that the addition of PSA (10^{-2}M) to the medium containing nicotinamide (10^{-6}M) resulted in growth inhibition that was reversed by the addition of higher amounts of niacin or niacinamide. On the other hand, it was reported that PSA and its amide had some growth stimulating action on *P. vulgaris* and *S. aureus* at a relatively high concentration (97). Similarly, complete replacement of the niacin requirement of *Shigellae* species by PSA (1 mg/ml) has been observed (92).

The mechanism by which PSA substitutes niacin is not clear. It is possible that there is either a conversion of PSA to niacin or that PSA itself is incorporated to yield a coenzyme analog which is biologically functional.

Wooley (126) observed that 3-acetylpyridine (3-AP) was a niacin antagonist in animals. 3-AP differs from niacin by the replacement of an acetyl group for the carboxyl group. Although this analog could not support the growth of a number of microorganisms, it did substitute niacin to some

extent for some niacin-requiring strains of dysentry bacilli (82). The toxicity of 3-AP in animals was shown to be due to the formation of abnormal coenzyme. For example, the formation of the 3-AP analog of NAD in the brain and in the spleen of mice was demonstrated after the administration of 3-AP (53, 54). In this case, the 3-AP replaced the nicotinamide moiety of NAD.

The 3-AP analog of NAD was the first one that was found to be active with a number of dehydrogenases. 3-Acetylpyridine-NAD (APNAD) was shown to serve as a coenzyme with yeast alcohol dehydrogenase at approximately 10% of the rate at which NAD served, while pyridine-3-aldehydeNAD (Py-3-NAD) reacted at a considerably slower rate. With horse alcohol dehydrogenase or with glutamate dehydrogenase, both APNAD and Py-3-NAD reacted more rapidly than the normal NAD (53, 54).

A large number of compounds related to dipicolinate, an analog of niacin, were investigated for their ability to replace dipicolinate in restoring sporulation in a mutant strain of *Bacillus megaterium*. Of the compounds tested, only 4-H-pyran-2, 6-dicarboxylic acid and dipicolinic acid-N-oxide were effective in restoring sporulation and heat resistance. However, 4-H-pyran-2, 6-dicarboxylic acid was found to be more effective than the natural compound, dipicolinate, in these respects (31, 32).

In animals, 6-aminonicotinamide (6-AN) has been shown to be a potent analog of niacin. The toxic effects of 6-AN upon

injection into animals are due to its incorporation into analogs of NAD and NADP (52). Formation of 6-AN-NAD has also been demonstrated *in vitro* (21, 22) and it is a competitive inhibitor of several NAD- and NADP-dependent enzymes (17). Dietrich et al. (22) studied the effect of 6-AN administration on the activities of several NAD linked enzyme systems in animal adenocarcinoma and found that the activity of lactate dehydrogenase was unaffected by the coenzyme analog, while 3-phosphoglyceraldehyde dehydrogenase and α-ketoglutarate oxidase activities were markedly inhibited. Concomitantly, there was a significant decrease in the adenine nucleotide levels in tumor tissue as well as in other organs. Decreased levels of NAD and NADP following the administration of 6-AN, were also observed in ascites tumor cells (12).

There was a considerable decrease in the release of $^{14}CO_2$ from glucose-1-^{14}C, when 6-AN treated animal tissues were used for the metabolic studies (40, 41). This and other data indicated that there was an inhibition of the two initial NADP-dependent enzymes of the HMP pathway *in vivo*. The inhibition was shown to be more significant at the 6PGD level than at the G6PD level. This was based on the finding that larger amounts of 6-phosphogluconate were accumulated in the brain of 6-AN treated animals compared to glucose-6-phosphate.

various other biochemical reactions outside the HMP pathway, are also inhibited by 6-AN. For instance, it has been reported that there was an inhibition in the incorporation of glucose intermediates into DNA, RNA and proteins. However, these adverse effects were probably secondary to the inhibition of the HMP pathway activity (64).

The effects of niacin analogs on the growth of various niacin-requiring microbes in a synthetic medium containing minimal amounts of niacin, were investigated (114). The results of these studies indicated that the growth of streptococci, E. coli and Lactobacillus plantarum were completely inhibited by 5-fluoronicotinic acid added a concentration of 0.05 to 1.0 μg/ml, but this concentration failed to inhibit the growth of S. aureus. However, 5-fluoronicotinamide in the same concentration was most inhibitory to the growth of S. aureus, E. coli and of streptococci. The growth of L. plantarum, S. aureus, P. vulgaris and of E. coli was inhibited by five halogenated nicotinic acid derivatives in the following order of decreasing effectiveness: 5-fluoro-, 5-bromo-, 2-fluoro- and 6-fluoronicotinic acid (47). Such inhibition of microbial growth was reversed by the addition of niacin, niacinamide or NAD to the growth medium. According to Streightoff (114) replacement of the 5-fluoro group with an amino, bromo, chloro, dimethylamine, hydroxyl or other

groups resulted in loss of inhibition of growth of strepto-cocci. Changing the fluoro group from 5th to 6th position on the pyridine ring also resulted in a compound with very little inhibitory activity. In comparison when amino group was on the 4th or 6th position the compound exhibited significant growth inhibitory activity (114).

Since there are contradictory reports in the literature on the growth effects of these niacin analogs, studies were undertaken to explore their effects on the growth of S. aureus. Niacin analogs such as 3-acetylpyridine and 6-aminonicotinamide have been studied extensively in animal system and their toxic effects have been attributed to changes in the glucose catabolic pathways and in enzyme and coenzymes involved in these pathways. Therefore, it was of interest to investigate the effect of these analogs on the glucose catabolic pathways and on the coenzymes in S. aureus. This would give us an excellent oppertunity to study the relation of the glycolytic enzymes, nicotinamide coenzymes NAD(P) or analogs of these coenzymes to the glucose catabolic pathways.

II. MATERIALS AND METHODS

Bacterial cultures. Most of the experimental work was performed with *Staphylococcus aureus*, strain Towler, originally obtained from Dr. W. C. Noble, St. Mary's Hospital Medical School, London, England. Strain 18Z was received from Dr. F. Kapral, Ohio State University, Columbus, Ohio, while strain 23 was received from Dr. S. Cloutier, University of Montreal, Canada. *S. aureus* serotypes 1 to 4 and *S. epidermidis* ATCC 115 and 12228 were obtained from Dr. W. W. Yotis of this department. Strains of *S. flexneri*, *P. vulgaris* and *P. morgani* were obtained from the departmental culture collection.

The stock cultures of the organisms were maintained on 4% Trypticase soy agar (Baltimore Biological Laboratories, Baltimore, Maryland) at 4 C and were transferred to fresh slants every 2-3 weeks.

Media

a. Preparation of liquid media. Vitamin-free Casitone (Difco Laboratories, Detroit, Michigan.) was used extensively throughout the studies. The medium was prepared in 2% concentration in distilled water and dispensed in 100 ml aliquots into specially cleaned 250 ml Erlenmeyer flasks. All flasks were cleaned by boiling in distilled water containing 2% (V/V) Fl-70 Biograde detergent(Fisher Scientific, Pittsburg,

pa.) and rinsed several times with distilled water. This step was necessary in order to eliminate any traces of vitamins remaining in the glassware. The medium was sterilized by autoclaving at 121 C for 15 min. Sterile vitamin solutions were added aseptically to these flasks, whenever needed. The preparation and concentration of the vitamins used are described later.

<u>Preparation of synthetic medium</u>. The synthetic medium of Idriss and Blumenthal (48) was prepared and sterilized by membrane filtration (type HA, 0.45 μ pore size, Millipore Filter Corp., Bedford, Mass.). Autoclaving of the medium was avoided to prevent any degradation of labile components of the medium. The complete composition of the medium has been described by Idriss and Blumenthal (48).

<u>Preparation of vitamins and niacin analogs</u>. Niacin, thiamine and the niacin analogs were separately prepared and sterilized by membrane filtration. For the sterilization of the vitamins or analogs, sterile Millipore 'Swinnex' filters containing a membrane (0.45 μ pore size) were used. Stock solutions of the vitamins and niacin analogs were prepared in deionized distilled water in various concentrations and were stored at 4 C for not more than 2 weeks. The vitamins or the analogs were added aseptically to the sterile medium when it was cool.

Growth conditions. In all experiments the starter culture was prepared in the following manner. A loopful of cells from the stock culture was plated on a Trypticase soy agar plate and incubated at 37 C for 18-24 hr. A loopful of these freshly grown cells was then inoculated into a starter flask. The medium for the starter cultures was 100 ml of 2% (W/V) Vitamin-free Casitone (Difco), which was dispensed into 250 ml Erlenmeyer flasks and sterilized at 121 C for 15 min prior to inoculation. Once inoculated, these flasks were incubated for 6 to 8 hr at 37 C on a rotary shaker. The use of freshly grown cells to inoculate the starter flask was necessary to uniformly obtain growth of the culture in the medium within this time period.

The growth medium was inoculated with 1 ml of inoculum from the starter culture medium (Vitamin-free Casitone). The flasks were then incubated for 17 hr at 37 C on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.) at 240 rev/min. The cells were harvested, usually near the end of the log phase (17 hr) by centrifugation at 4 C in 250 ml centrifuge bottles (IEC) at 30,000 x g for 15 min. The cell pellet was collected, washed twice with 0.8% saline and then used for the experiments.

To inoculate the synthetic medium, the cells from the starter culture medium were first centrifuged at 30,000 x g for 10 min, washed twice with sterile 0.8% saline, adjusted to the standard optical density, 1500 Klett units; (a 1:10 dilution reads 150 Klett units) and then one ml was transferred to the synthetic medium. The flasks were then incubated for 17 hr at 37 C.

Growth measurements. Growth was determined by measuring the turbidity of the culture in nephelometer flasks with a Klett-Summerson Colorimeter provided with a 600 nm filter. Sterile Vitamin-free Casitone or synthetic medium was used as a blank. The nephelometer flasks were 300 ml Erlenmeyer flasks with a side arm tube attached to the flask. The flask did not contain more than 100 ml of the medium and were exclusively used for the measurement of growth. The turbidity measurements were performed rapidly so as to provide minimum exposure of the cells to environmental changes. All the experiments were run in duplicate flasks and the values are the average of two separate experiments. When the difference in turbidity readings between duplicate samples was greater than 25%, the results of the experiment were rejected.

Vitamin contents of the media. 2% Vitamin-free Casitone medium contained following concentrations of niacin and thiamine, based on information provided to us by Difco Laboratories:

Niacin .... 0.013 μg/ml

Thiamine ..... <0.00013 μg/ml

Determination of glucose catabolic pathways.

a. Radioisotope experiment. At the end of 17 hr of incubation the cells were harvested by centrifugation at 30,000 x g for 10 min at 4 C, washed twice and suspended in potassium phosphate buffer (0.1 M, pH 7.0). Each suspension was then adjusted with buffer to the same turbidity (180 Klett units using a 1:10 dilution) using a 600 nm filter. A 1:10 dilution of this heavy cell suspension that gave a reading of 180 Klett units was approximately equal to 3 mg dry weight/ml, an amount which was sufficient for the experiments.

Metabolic flasks inoculation. Radioisotope experiments using labelled glucose were performed according to the method described by Goldman and Blumenthal (35). Erlenmeyer flasks (50 ml) fitted with a center well (metabolic flask) were used throughout the experiments. The center well was capable of holding a small vial which contained a paperwick and also kept

the vial isolated from the surrounding medium.

Employing a 1 ml long tip pipet, 0.5 ml of 0.5 M glucose-1-^{14}C was added to the main compartment of each of the two flasks. Similarly, glucose-6-^{14}C of same concentration (0.5 M) was added to the two additional flasks. One ml of 0.1 M (pH 7.0) potassium phosphate buffer was then added to the main compartment of all flasks using a 5 ml long tip pipet. For each metabolic flask in the experiment, two serological tubes were prepared containing 2.5 ml of 10% (V/V) perchloric acid. These were used for quantitative determination of glucose in the supernatant fluid. Using a 10 ml pipet, 2.5 ml of the standard cell suspension was then added to the main compartment of each of the series of flasks at one min intervals. During each one min interval zero hour samples were removed for glucose as follows: the glucose-bacterial suspension in the flask was well mixed for 10 to 15 sec, and then a 0.5 ml sample was removed using a separate 1 ml long tip pipet for each flask, and added to a serological tube containing perchloric acid. Following the removal of the zero hour sample, a small glass vial containing a folded piece of Whatman No. 1 filter paper (to increase CO_2 absorption) was added to the center well using a forcep. Each flask was immediately sealed with a serum stopper and was incubated in a Dubnoff metabolic shaker

at 37 C. The flasks were shaken at about 100 cycles/min for 120 min.

After all of the flasks had been started, a 1 ml syringe with a long 22 gauge needle was used to inoculate 1 ml of the CO_2 absorbing solution into each vial previously placed in the center well. This solution consisted of ethanolamine ethylene glycol monomethylether of the following composition (51): ethanolamine - ethyleneglycol monomethylether (1:2 V/V).

<u>Liquid scintillation counting of $^{14}CO_2$.</u> When the incubation period was over, the flasks were removed from the shaker in the same order as they were put into the water bath. When the flask was removed, the serum stopper was taken off, and the entire center well vial with its contents (trapped CO_2, CO_2 absorbing solution and the paper) was carefully added to a 20 ml scintillation vial, of low potassium content (Packard Instrument Co.). At the same [illegible] 0.5 ml of sample from the main compartment of the vessel was removed for analysis of glucose remaining in the [illegible], and added to the second serological tube containing [illegible] acid.

[illegible] scintillation vial, containing a center vial, [illegible] 1[illegible] ml of a mixture of toluene, ethylene-

glycolmonomethylether, 2,5-diphenyloxazole (PPO) (1:2:0.181 V/V/W) as scintillator and 1 ml of ethanolamine-ethyleneglycol monomthylether. A background scintillation vial was included in the experiments which consisted of non-incubated center well vial with a filter paper and a scintillation mixture of the same composition which was used to count the samples. Plastic screw caps lined with tin foil were then carefully placed on the scintillation vial. All of the vials were well mixed, cleaned on the outside, and then placed in Packard Tricarb Scintillation Spectrometer Model # 3320, for counting. All the vials, which were cooled for 6 to 8 hr before the counting started, were counted at least 3 times. The system had a counting efficiency of approximately 80% for ^{14}C.

Determination of glucose utilization. The serological tubes containing bacterial-glucose suspension were centrifuged at 30,000 X g for 15 min. Following the removal of bacterial cells, glucose was determined by the anthrone method of Seifter et al. (106).

b. Estimation of the pathways of glucose catabolism. The pathways of glucose catabolism were estimated using the basic mathematical formulae devised by Wang et al. (119) and as modified slightly by Blumenthal (5).

From the information of the amount of radioactive $^{14}CO_2$ yield from glucose-1-^{14}C and -6-^{14}C along with the quantity of glucose (-1-C and -6-C) metabolized, determined by the anthrone method, the percentage of HMP and EM were calculated using the following formulae. The percentage of $^{14}CO_2$ yielded by glucose-6-^{14}C is a rough estimation of the extent of the operation of the TCA cycle.

$$HMP = \frac{G_1 - G_6}{G_T} \times 100$$

where G_1 = $^{14}CO_2$ coming from glucose-1-^{14}C (cpm)

G_6 = $^{14}CO_2$ coming from glucose-6-^{14}C (cpm)

G_T = Total amount of glucose labelled utilized by cells during incubation.

%EM = 100 - %HMP

<u>Phosphate buffer-glucose medium</u>. In order to study the effects of niacin or niacin analogs under resting cell conditions, buffered glucose medium, devoid of any nitrogen source, was prepared by dissolving glucose in 0.05 M potassium phosphate buffer, pH 7.0, to yield a final concentration of 50 μmoles/ml. The medium was sterilized by autoclaving at 121 C for 10 min. Niacin, thiamine or niacin analogs were added to this medium under aseptic conditions. Washed cells, previously grown in Vitamin-free

Casitone (VFC) or VFC containing niacin or niacin and thiamine were inoculated in this medium and incubated at 37 C for 2 to 3 hr. The incubated cells were then used either for isotope experiment or for enzyme analyses.

Enzyme analyses of S. aureus.

a. Preparation of cells for enzyme extraction. Levels of glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) and of other enzymes from the HMP pathway and the level of isocitrate dehydrogenase (ICD) from the TCA cycle, were investigated. Staphylococcal cells were grown in Vitamin-free Casitone or in synthetic medium alone or in the presence of the vitamins. All cultures were grown at 37 C on a rotary shaker until late in the log phase, ordinarily 17 hr. This period of growth was necessary because high cell densities were needed in order to obtain sufficient amounts of cells.

At the end of the growth period, the staphylococci were harvested by centrifugation at 30,000 x g for 15 min using a refrigerated centrifuge (International Equipment Co., Model B-20) at 4 C. The supernatant fluid was carefully discarded and the cells were then washed twice with 0.8% saline. The cell pellet (ca. 1 g weight) was then suspended in approximately four volumes of 0.01 M tris (hydroxymethyl)-aminomethane (tris) buffer, pH 7.5, containing 0.0001 M EDTA (ethylenediamine tetraacetic acid).

The suspension was vigorously mixed with a vortex mixer and then subjected to sonic treatment in a 10 Kc Branson Sonic oscillator (Model S-75) as follows. A 3.8-cm (diameter) solid step horn terminating in a 1.3-cm (diameter) irradiating area (stainless steel) attached to 500 ml water jacketed chamber was used for sonication of the cells. The cell suspension was cooled to 4 C prior to the treatment and was subjected to sonic treatment for a total of 15 to 20 min. The sonication was performed intermittently, never more than 40-50 sec at a time, and was followed by a 1-2 min period of cooling in order to prevent a rise in temperature and loss of enzymatic activity. During the treatment time cooling water (-10 C) was circulated through the jacketed chamber. Treatment times longer than 20 min tend to result in loss of enzyme activity of the extracts. Sonic treatment of 17 min was found to be optimum for breakage of the staphylococcal cells.

After sonic treatment the mixture was centrifuged at 30,000 x g for 20 min at 4 C. The supernatant fluid was carefully decanted into a test tube, which was stored in an ice bucket, and then used for the enzyme assay.

Before enzyme analysis, the protein concentration in the crude extract was determined by the method of Waddell (118). This procedure of protein determination which depends on the absorption of peptide bonds at 215 and 225 nm, has a

greater sensitivity than the other methods and eliminates the need for the addition of any reagents for color development.

b. Protein determination. A 0.02 to 0.04 ml sample of crude extract was added to a quartz cuvette and deionized distilled water was added to yield a final volume of 1.5 ml. The blank cuvette contained deionized distilled water. Absorbancy was recorded at 215 and 225 nm and the difference of optical density (OD) was multiplied by the factor of 144 and by the dilution factor. This yielded the final protein concentration in μg/ml of enzyme extract.

The crude extracts were stored at -15 C and showed very little loss of activity of the enzymes studied, even after storage for several months.

Reagents for the enzyme assay. All the solutions were made using doubly distilled, deionized water.

1. NADP solution (3 μmoles/ml)

   Dissolve 4.6 mg of NADP powder in 2 ml water

2. NAD solution (3 μmoles/ml)

   Dissolve 4.0 mg of NAD powder in 2 ml water

3. Tris-HCl buffer (75 μmoles/ml)

   Dissolve 0.09075 g of Tris (hydroxymethyl-aminomethane) in 100 ml of water. Adjust the pH to 7.5 with 1 N HCl.

4. Manganese sulfate (30 μmoles/ml)

Dissolve 0.830 g of $MnSO_4$, H_2O in water and make up 100 ml.

5. Isocitrate (30 μmoles/ml)

   Dissolve 7,75 mg of d-isocitrate-Na_2 in 1 ml water.

6. Glucose-6-phosphate (30 μmoles/ml)

   Dissolve 15.6 mg of glucose-6-phosphate-Na_2 in 2 ml water.

7. 6-Phosphogluconate (30 μmoles/ml)

   Dissolve 20.5 mg of 6-phosphogluconate-Na_2 in 2 ml water.

8. NADH solution (1 μmole/ml)

   Dissolve 3.4 mg in 5 ml 0.01 M Tris buffer, pH 8.0. NADH solution should be kept for no more than one week because of the possible formation of an inhibitory factor.

9. NADPH solution (1 μmole/ml)

   Dissolve 4.2 mg in 5 ml of 0.01 m Tris buffer, pH 8.0. Keep for no more than one week.

10. KCN solution

    Dissolve 1.3 mg in 10 ml water.

11. AcPyAD solution

    Dissolve 12.5 mg in 1 ml water.

12. Glucose-6-phosphate dehydrogenase, (G6PD) (1 mg protein/ml)

    Dilute the stock solution with 2.2 M ammonium sulfate solution.

13. Isocitrate dehydrogenase (ICD) (1 mg protein/ml)
Dilute the stock solution with 2.2 M ammonium sulfate solution.

Enzyme assays. All the enzyme assays were performed at 25 C on Gilford spectrophotometer, model 2000 (Gilford Instrument Laboratory, Oberlin, Ohio.) equipped with automatic recorder. Standard 1.5 ml and 3.0 ml cuvettes (quartz) with 1.0 cm light path were used. Enzyme assays were performed as described by Ragland et al. (96).

a. Assay for isocitrate dehydrogenase. The assay of isocitrate dehydrogenase was based on the reduction of NADP to NADPH.

$$\text{Isocitrate} \xrightarrow[\text{NADP} \qquad \text{NADPH}]{CO_2} \text{a-ketoglutarate}$$

To a 1.5 ml cuvette the following solutions were added: Tris buffer 75 μmoles, $MnSO_4$ 1.5 μmoles, isocitrate 3 μmoles, deionized water 0.25 ml, extract 0.05 ml. After the determination of initial absorbance (at 340 nm), the reaction was started by adding 0.15 μmoles of NADP and was followed by recording absorbancy at 340 nm at 30 sec interval. The blank cuvette contained all the ingredients except NADP, which was replaced by distilled water.

b. Assay for glucose-6-phosphate dehydrogenase.

glucose-6-phosphate --------→ 6-phosphogluconate
NADP NADPH

The conditions for the assay of G6PD were the same as those for ICD, except that isocitrate was replaced by glucose-6-phosphate (3 μmoles/0.1 ml).

c. Assay for 6-phosphogluconate dehydrogenase.

6-phosphogluconate ---------→ ribulose-5-P + CO_2
NADP NADPH

The conditions for the assay of G6PD were the same as those for ICD, except that isocitrate was replaced by 6-phosphogluconate (3 μmoles/0.1 ml).

d. Assay for transhydrogenase. The transhydrogenase assay was based on the reduction of AcPyAD.

NADPH + AcPyAD ------------- NADP + AcPyADH

To a 1.5 ml cuvette the following solutions were added: K_2HOP_4, 75 μmoles; $NADPH_2$, 0.45 μmoles; KCN, 1.5 μmole; distilled water, 0.30 ml; and crude extract 0.05 ml. After the determination of the initial absorbance, the reaction was started by addition of 0.9 μmoles of AcPyAD and was followed by recording at 30 sec intervals the absorbance at 375 nm. The blank cuvette contained all ingredients except AcPyAD, which was replaced by distilled water.

e. Assay for NADH and NADPH oxidase. To a 1.5 ml cuvette the following solutions were added: K_2HPO_4, 75

μmoles; NADH or NADPH, 0.05 μmoles; and distilled water, 0.4 ml. The reaction was started by adding 0.05 ml of extract and was followed by observing the decrease in absorbance at 340 nm.

Calculations. All specific activities are expressed as nanomoles of coenzyme reduced/mg of protein/min.

$$\text{Specific activity} = \frac{\Delta E \times V}{10^{-6} \times \varepsilon x d \times \text{mg of protein in reaction mixture.}}$$

ΔE = change in absorbance/min.

V = volume of the reaction mixture in the cuvette.

ε = Extinction coefficient of the light absorbing substance. ($cm^2/\mu mole$) for NAD (340 nm)..6.22×10^6 for AcPyAD (375 nm) .. 5.2×10^6

d = light path of cuvette (1 cm).

Uptake of niacin and its analogs.

a. Preparation of staphylococcal cells for uptake studies. Staphylococci previously grown in unsupplemented Vitamin-free Casitone were harvested by centrifugation, as described previously. These cells were washed three times with sterile saline (0.09%) and then suspended in 0.01 M potassium phosphate buffer (pH, 7.0) to yield a cell suspension of known concentration. The concentration of the cells was adjusted to a turbidity of 1500 Klett units

(150 Klett units on a 1:10 dilution) using distilled water as a blank.

b. <u>Measurement of uptake of niacin and niacin analogs.</u> The incubation medium for the uptake studies was of the following composition:

Potassium phosphate buffer, pH 7.0, 0.1 M ..	1.0 ml
Glucose (1%)	0.5 ml
Niacin or niacin analog (conc. variable) ...	1.0 ml
Washed cells	2.5 ml

The cell suspensions and the reaction mixture were equilibrated at 37 C before mixing. The reaction was started by the addition of labelled niacin or labelled niacin analog. Unless otherwise stated, the flasks were incubated at 37 C with gentle shaking to secure good mixing of the cells with the medium. All incubations were carried out in duplicate and the experiments were repeated at least once. The concentrations of niacin, quinolinic acid and of isoniazid that were used, are listed in the respective tables of the results section.

The flasks were incubated for various intervals of time. When the incubation had been completed, 0.2 ml samples were rapidly withdrawn and filtered on membrane filters (0.45 μ pore size) that had been previously saturated with the appropriate unlabelled substrate. The cells which were retained by the filter were quickly washed twice with 1 ml deionized distilled water. The filtrate and the membrane filter containing the cells were added to separate 20 ml

scintillation mixture to each vial, they were counted in a liquid scintillation spectrometer. When the difference in counts between duplicate samples was greater than 25%, the results of the experiment were rejected.

The scintillation mixture used for counting radioactivity in the cells and in the filtrate was of following composition: p-dioxane, 900 ml; anisole, 150 ml; 1,2-dimethoxyethane, 150 ml; PPO, 18g; and POPOP, 60 mg.

Among the factors that were included in the permeability studies were the effects of time, temperature, pH and energy source, competition between niacin and its analogs, and the rate of exchange of labelled niacin.

c. Competition studies between niacin and its analogs. Competition studies between niacin and its analogs were performed using radioactive niacin and either unlabelled niacin or unlabelled analog. The concentration of unlabelled niacin or unlabelled analog (pyridine-N-oxide, isoniazid, quinolinic acid, or 3-pyridylcarbinol) added to the incubation mixture, was 1000-fold higher than the concentration of labelled niacin. The reaction was carried out for 1 hr period after which 0.2 ml of samples were withdrawn for the estimation of radioactivity within the cells and in the supernatant fluid.

d. Measurement of exchange between intra- and extracellular niacin and its analogs. In the experiment involving exchange between accumulated radioactive niacin and extracellular unlabelled niacin or the analogs, staphylococcal cells were incubated in reaction mixture containing niacin (2 μg/ml), as described previously, for 60 min at 37 C. During this incubation period the niacin binding sites became saturated. These niacin loaded cells were collected by centrifugation at 30,000 ×g for 15 min at 4 C. They were washed twice with sterile 0.8% saline and suspended in 0.05 M phosphate buffer, (pH 7.0). The cell density was adjusted to 3.5 mg of dry weight/ml, as described previously. Exchange reactions were initiated by adding standardized cell suspension to incubation medium from which labelled niacin was omitted and to which un-labelled niacin (2 mg/ml) or one of the analogs (2 mg/ml) was added. After the desired intervals of time, samples were removed and cellular radioactivity was determined.

Chemicals

Chemicals and enzymes. The chemicals and enzymes were obtained from commercial sources and were not further purified. β-NAD (yeast), NADP (from yeast NAD), β-NADH (grade III, yeast), NADPH (type III), AcPyAD (type I), glucose-6-phosphate dehydrogenase (Zwischenfement, type V), isocitrate dehydrogenase (type IV), chloramphenicol and

puromycin were purchased from Sigma Chemical Co., St. Louis, Mo. The disodium salts of glucose-6-phosphate and isocitrate were obtained from C. F. Boehringer, Mannheim, Germany. Thiamine hydrochloride was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J., and niacin (nicotinic acid) from Merck and Co., Rahway, N.J. Anthrone was a product of Nutritional Biochemical Corp., Cleveland, Ohio., and actinomycin D was purchased from Mann Research Laboratories, N.Y. All other chemicals were reagent grade.

Chemicals for scintillation mixtures. PPO (2,5-diphenyloxazole), anisole, 1,2-dimethoxyethane and ethanol amine were purchased from Eastman Organic Chemicals, Rochester N.Y. Ethyleneglycolmonomethylether and p-dioxane were obtained from Fisher Scientific Co., Fairlawn, N.J. POPOP (1, 4-bis-(2-(5-phynyloxazolyl))-benzene was purchased from Packard Instrument Co., Downers Grove, Ill.

Niacin analogs. Nicotinuric acid, pyridine-3-sulfonic acid and picolinic acid were obtained from Nutritional Biochemical Co., Cleveland, Ohio. The halogenated derivatives of niacin, 5-fluoro-, and 5-chloronicotinic acid, and 5-fluoronicotinamide were gifts from Dr. F. Streightoff, Eli Lily Co., Indianapolis, Ind. Hydroxylated derivatives of niacin, 2-hydroxy, 4-hydroxy and 5-hydroxyniacin were kindly provided by Dr. O. N. Miller of Hoffman-La Roche, Nutley, N.J. All other niacin analogs were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin.

Radiochemicals. Niacin-7-^{14}C was purchased from Nuclear Chicago Corp., Des Plaines, Illinois, and had a specific activity of 27.9 mCi/mM. Quinolinate-^{14}C and isoniazid-^{14}C were purchased from G. D. Searle and Co., Des Plaines, Illinois, and had a specific activity of 43.7 mCi/mM and 9.85 mCi/mM, respectively. Glucose labelled on -6-^{14}C and -1-^{14}C were obtained from Volk Radiochemical Co., Des Plaines, Illinois.

III. RESULTS

A. Growth of *S. aureus* in Vitamin-free Casitone.

S. aureus requires niacin for growth, and both niacin and thiamine are necessary for maximum growth stimulation. A typical growth curve of *S. aureus* strain Towler, in 2% Vitamin-free Casitone medium is shown in Fig. 1. This medium was chosen for the present studies because it contains very low amounts of niacin and thiamine.

The results of the growth curve (Fig. 1) demonstrated that there was only a moderate growth of staphylococci (i.e., 72 Klett units) in the absence of any exogenous addition of the vitamins, and even this amount of growth was due to traces of vitamins present in the medium. The amounts of vitamins present in 2% Vitamin-free Casitone are as follows: 0.013 μg/ml of niacin, and <0.00013 μg/ml of the thiamine (Personal communication, Difco Laboratories). Addition of thiamine (2 μg/ml) to the growth medium increased the amount of growth only slightly above that in an unsupplemented control. The addition of 2 μg/ml of niacin, however, increased the turbidity readings from 72 to 250 Klett units (24 hr). This stimulation indicated that niacin was present in limited concentrations for growth in the unsupplemented growth medium. Supplementation with both niacin and thiamine resulted in maximal increase of the growth rate.

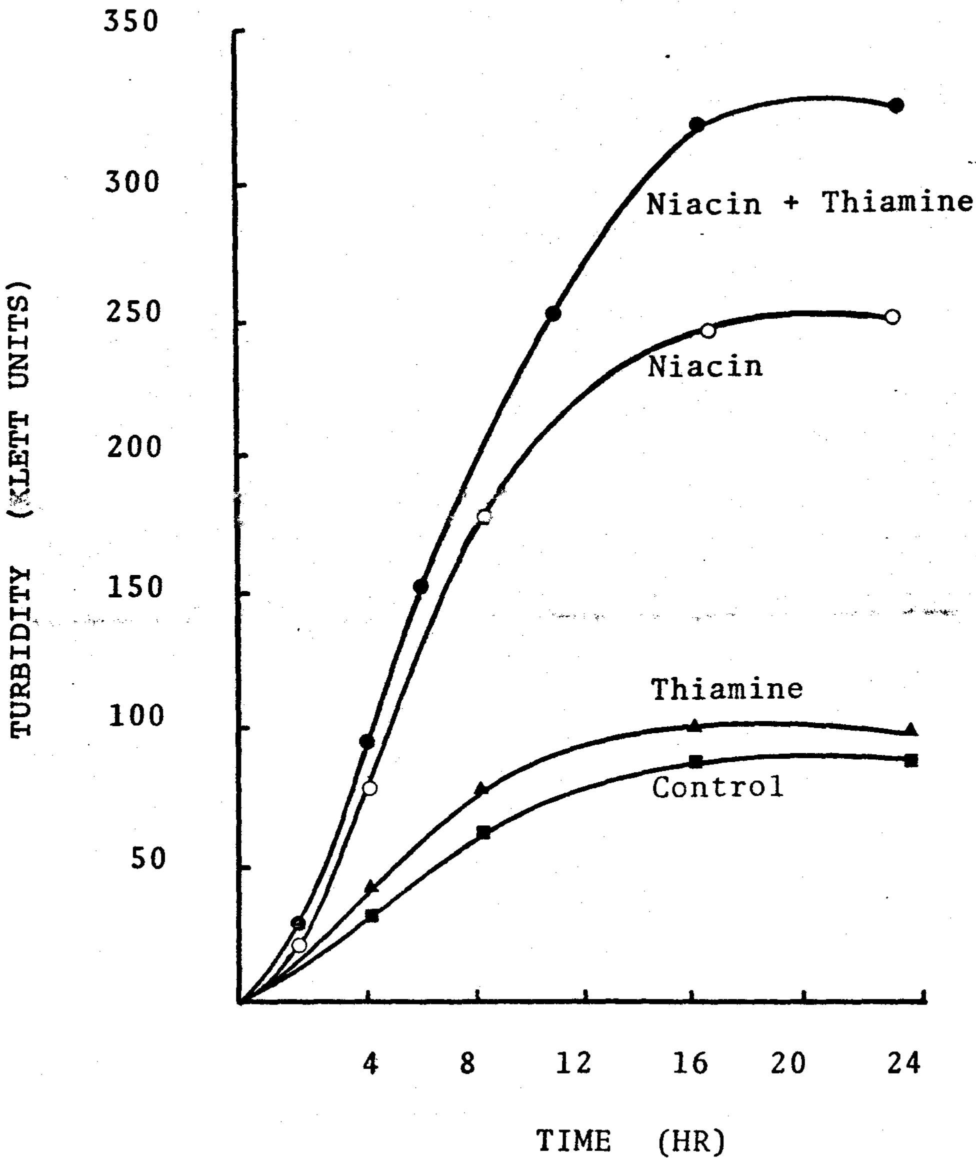

Figure 1. Growth of S. aureus Towler, in 2% Vitamin-free Casitone with or without supplementary niacin(2 μg/ml) and/or thiamine(2 μg/ml). The growth, on rotary shaker at 37 C, was followed by measuring the turbidity at 600 nm.

After 24 hr of incubation, the turbidity measurement reached a maximum of 336 Klett units, as compared to 72 Klett units in the unsupplemented Vitamin-free Casitone. These results demonstrated necessity for the presence of both vitamins for maximal stimulation of staphylococcal growth.

B. Analyses of the glucose catabolic pathways and of enzyme levels.

(1) Glucose catabolism in Vitamin-free Casitone and in synthetic medium.

Table 1 shows the result of isotopic experiments when vitamin supplements were added to a coagulase positive strain of *S. aureus* strain Towler, grown in 2% Vitamin free Casitone and in synthetic medium. The results demonstrated that staphylococci grown in unsupplemented 2% Vitamin-free Casitone had a very low metabolic rate and a markedly decreased utilization of the HMP pathway. Approximately 5% of the glucose was catabolized via the HMP pathway, while the relative activity of the TCA cycle was less than 1%. Addition of thiamine alone to the growth medium did not stimulate the HMP cycle and resulted in only a small increase in the TCA cycle activity. When the cells were grown in a niacin-supplemented medium, however, they exhibited a 3- to 4-fold increase in the HMP pathway (i.e., from 5 to 22%) with a slight stimulation of the TCA cycle activity. Supplementation with vitamins resulted in a 3-fold increase

TABLE 1

Effect of niacin and thiamine on the glucose catabolic pathways of *Staphylococcus aureus* strain Towler

Addition (μg/ml) to 2% Vitamin-free Casitone	% Glucose[a] catabolized via EM	% Glucose[a] catabolized via HMP	TCA cycle activity (%)
None	95.0	5.0	0.9
Thiamine (2)	94.8	5.2	1.2
Niacin (2)	78.0	22.0	1.0
Niacin(2) + Thiamine (2)	82.0	18.0	4.5
Addition (μg/ml) to synthetic medium			
Niacin (8)	67.0	33.0	2.0
Niacin (8) + Thiamine (4)	65.0	35.0	4.0

[a] Glucose catabolic pathways (EM and HMP) and the TCA cycle activity were determined by the yield of $^{14}CO_2$ from glucose-1- and -6-^{14}C.

The values are the average of at least 3 determinations.

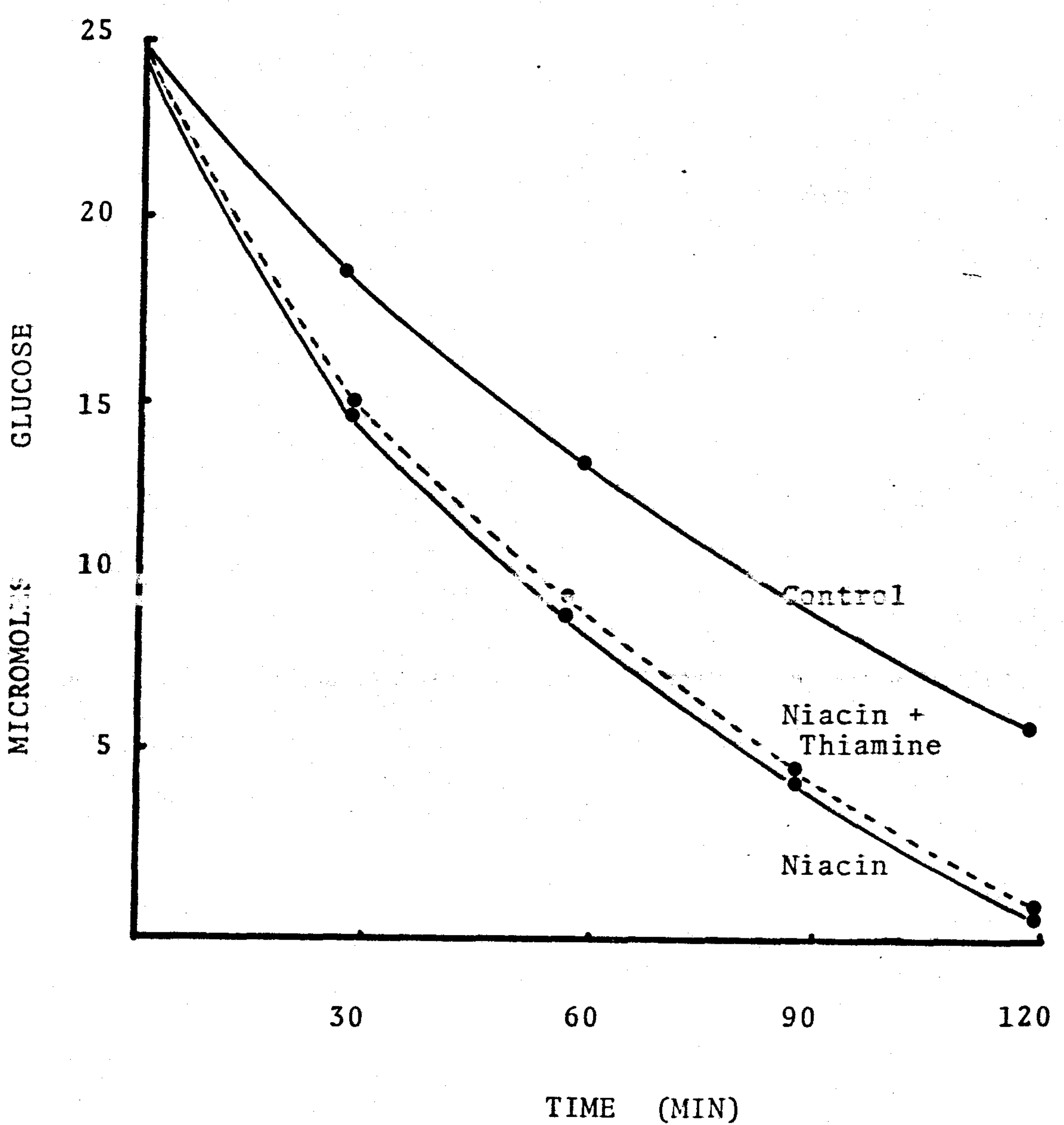

Figure 2. <u>Glucose utilization by *S. aureus*.</u> Staphylococcal cells were first grown in 2% Vitamin-free Casitone with or without supplementary niacin, 2 µg/ml, and/or thiamine, 2 µg/ml. The utilization of glucose was determined under resting cell conditions at indicated time by the method of Seifter et al. (106).

in utilization of the HMP pathway and a 5-fold increase in the activity of the TCA cycle. For instance, the amounts of glucose catabolized via the HMP pathway and the relative activity of the TCA cycle were 18% and 4.5%, respectively.

These experiments indicated that niacin was required for stimulation of the HMP pathway, while both niacin and thiamine were necessary for the maximal operation of the TCA cycle. Increasing the concentration of the vitamin in the medium from 2 to 10 μg/ml did not result in any additional changes in the degree of utilization of the different glycolytic pathways.

Results of experiments with cells grown in synthetic media were more or less similar to the results obtained with cells grown in Vitamin-free Casitone. Since staphylococci were unable to grow in synthetic media lacking niacin, the isotope experiments were not done. The amount of glucose catabolized via the HMP pathway was approximately 30-35% in cells grown in the synthetic medium compared to 18-20% in cells grown in supplemented Vitamin-free Casitone. The TCA cycle activity in the synthetic medium was increased (2%), even in the absence of added thiamine. However, addition of thiamine to the niacin-containing synthetic medium resulted in a 2-fold increase (4%) in the TCA cycle activity. This indicated that greater quantities of

thiamine were needed for maximal operation of the TCA cycle than were needed for the maximal operation of the HMP pathway.

(2) <u>Enzyme levels of the glycolytic pathways of staphylococci previously grown in Vitamin-free Casitone and in synthetic medium with or without vitamin supplementation.</u>

The growth of *S. aureus* was relatively poor in unsupplemented Vitamin-free Casitone, and the relative activities of the HMP pathway and the TCA cycle were also low. The addition of niacin or niacin and thiamine to the growth medium not only stimulated growth maximally (Fig.1) but also increased activities of the HMP and the TCA cycle by 4-fold (Table 1). Analyses of some key enzymes of the HMP pathway and of the TCA cycle under various conditions of growth in both Vitamin-free Casitone and in synthetic media were undertaken in an attempt to determine if the increased activities of the pathways were due to increased enzyme synthesis.

When *S. aureus* was grown in Vitamin-free Casitone medium (Table 2), the specific activities of G6PD ranged from 40 to 50 nmoles/mg protein/min, while the activity of 6PGD and ICD varied between 15 to 25 nmoles/mg protein/min. The addition of thiamine alone resulted in only a slight increase in ICD activity (from 15 to 21 units), while the activities of G6PD, 6PGD and of other enzymes remained unaffected.

TABLE 2

Specific activities of enzymes from extracts of *S. aureus* grown in various media

Growth Medium	Specific activity of enzyme, nmoles/mg protein/min					
	Glucose-6-P-dehydrogenase	6-Phosphogluconate dehydrogenase	Isocitrate-dehydrogenase	Transhydrogenase	NADPH oxidase	NADH oxidase
Vitamin-free Casitone	49	23	15	12	3	12
Vitamin-free Casitone + niacin (2 μg/ml)	161 (20.5[a])	37 (2.0)	22 (1.16)	14 (0.3)	6 (0.6)	25 (1.4)
Vitamin-free Casitone + thiamine (2 μg/ml)	48 (0.2)	20 (0.5)	21 (0.8)	7 (0.6)	3 (0.0)	16 (0.5)
Vitamin-free Casitone + niacin and thiamine	153 (13.7)	39 (2.7)	49 (5.6)	9 (0.35)	8 (0.9)	19 (0.7)
Synthetic medium + niacin (8 μg/ml)	75 (3.1)	20 (0.36)	16 (0.14)	3 (1.6)	3 (.0)	11 (0.13)
Synthetic medium + niacin (8 μg/ml) and thiamine (4 μg/ml)	98 (5.5)	30 (1.17)	28 (2.1)	8 (0.57)	5 (0.63)	5 (1.8)

Enzyme activities were determined by the method of Ragland et al. (96).

[a] Difference of mean by "t" test; a value of 2.0 or greater is significant at the 5% level or better.

Following growth stimulation brought about by the addition of niacin to the growth medium, however, there was a 3- to 4-fold increase in the specific activity of G6PD (from 49 to 161 units), with some increases in 6PGD, ICD and TH levels. For instance, the specific activities of 6PGD, ICD and TH in niacin-grown cells were 37, 22 and 14 nmoles/mg protein/min, respectively. The NADH oxidase activity was elevated by 2-fold. Similarly, addition of niacin and thiamine not only stimulated G6PD synthesis but also increased ICD activity from 15 to 49 nmoles/mg protein/min. Generally, the activities of transhydrogenase, NADH oxidase and NADPH oxidase varied somewhat under the growth conditions tested.

The specific activities of G6PD, 6PGD and ICD in supplemented 2% Vitamin-free Casitone was 153, 39 and 49 nmoles/mg protein/min,respectively, while in synthetic medium it was 98, 30 and 28 nmoles/mg protein/min, respectively (Table 2). Although there was 30 to 50% reduction in the specific enzyme activities when the staphylococci were grown in synthetic medium, the operation of the HMP pathway in these cells was higher than in the cells grown in supplemented Vitamin-free Casitone.

(3) Glucose catabolic pathways and levels of some dehydrogenases of different strains of S. aureus grown in supplemented Vitamin-free Casitone. In order to show that the observation made on the Towler strain of *S. aureus* was

TABLE 3

Uniformity in effect of niacin and thiamine on the glucose catabolic pathways and on some dehydrogenases of staphylococci grown in Vitamin-free Casitone

Strain	Turbidity (17 hr), Klett units	Glucose-6-P[a] dehydrogenase	6-P-gluconate dehydrogenase	Isocitrate dehydrogenase	% HMP[b]	TCA cycle[c] activity
Towler	340	153	39	49	18.1	5.2
18 Z	310	149	32	51	18.5	4.3
23	331	160	28	44	19.3	4.6

a Specific activities are expressed as nanomoles/mg protein/min

b % glucose catabolized via the HMP pathway as determined by the yield of $^{14}CO_2$ from glucose-1- and -6-^{14}C.

c Relative activity of the TCA cycle as determined by the yield of $^{14}CO_2$ from glucose-6-^{14}C.

not peculiar to that strain alone, the glucose catabolic pathways and the specific activities of G6PD and 6PDG from the HMP pathway and of ICD from the TCA cycle were also measured in other coagulase positive strains of staphylococci previously grown in supplemented Vitamin-free Casitone. The results of these studies showed that the other coagulase positive strains followed the same pattern as strain Towler. For instance, addition of both vitamins to the growth medium not only resulted in maximum growth (310 to 340 Klett units) but also increased glucose utilization via the HMP pathway and the TCA cycle activity to 19% and 4.5%, respectively. The specific activities of G6PD, 6PGD and ICD in the strains tested were approximately 150, 32 and 45 nmoles/mg protein/min, values similar to those obtained for the Towler strain.

Addition of vitamins to the growth medium stimulated growth maximally (340 Klett units) and increased the HMP and the TCA cycle activities to 20% and 5%,respectively. Along with the increases in the HMP pathway, the level of G6PD was also tripled. Thus there was a strong correlation between the activities of the HMP enzymes and the degree of operation of the HMP pathway.

(4) Glucose catabolic pathways and enzyme levels under resting cell conditions. The effect of vitamins on the glucose catabolic pathways was tested under resting cell

conditions and the results are shown in Table 4. The data from Table 4 (Expt.I) indicated that the incubation of vitamin-deficient resting cells in buffered glucose resulted in cells which oxidized glucose in a manner similar to that of cells grown in the presence of the vitamins. For instance, when vitamin-deficient cells were incubated with the vitamins, the amount of glucose catabolized via the HMP pathway and the relative activity of the TCA cycle were 18% and 5.6%, respectively, values similar to those of cells grown in the presence of the vitamins. These results indicated that the vitamins stimulated these metabolic activities even in the absence of growth.

The results of the enzyme analyses under resting cell conditions are presented in Table 5. The levels of G6PD and 6PGD were not increased by the presence of niacin, even though the HMP pathway activity was increased. Similarly, ICD activity remained low when both vitamins were added to the medium. For example, the specific activities of G6PD and ICD were 43 and 15 nanomoles under resting cell conditions compared to 153 and 49 nanomoles/mg protein/min when the cells were grown in the presence of these vitamins. In spite of the low activities of G6PD and ICD (i.e., 43 and 15 units) under resting cell conditions, there was a greater utilization of glucose via both the HMP pathway and the TCA cycle.

TABLE 4

Changes in the glucose catabolic pathways and in the TCA cycle activity in the absence of growth by incubation of vitamin-deficient *S. aureus* Towler suspensions with thiamine and/or niacin

Expt.	Additions to the vitamin-deficient resting cell suspension[a]	TCA cycle activity (%)	% Glucose catabolized via EM	% Glucose catabolized via HMP
1.	None	0.2	92	8
	Thiamine, 4 μg/ml	1.2	92	8
	Niacin, 8 μg/ml	0.6	79	21
	Niacin and thiamine	5.6	82	18
2.	Niacin (2 μg/ml) and thiamine (2 μg/ml)	4.0	80	20
	Niacin and thiamine with chloramphenicol (25 μg/ml)	3.0	84	16
3.	Niacin (2 μg/ml) and thiamine (2 μg/ml)	4.0	84	16
	Chloramphenicol (25 μg/ml), plus niacin and thiamine	4.0	86	14
	Chloramphenicol (50 μg/ml), plus niacin and thiamine	3.0	83	17
4.	None	0.6	95	5
	Chloramphenicol(50μg/ml)	0.9	95	5
	Chloramphenicol(100 μg/ml), plus niacin and thiamine	4.0	88	16

[a] After growth for 17 hr in 2% Vitamin-free Casitone, the cells were washed with and suspended in 0.05 M Na and K phosphate buffer, pH 7.0, at twice their original concentration. Glucose was added at a concentration of 50 μmoles/ml, and the flasks were placed on a rotary shaker for 3 hr at 37 C. These cells were then harvested and used in the usual manner.

TABLE 5

Influence of the addition of vitamins, in the absence of growth, on some enzyme activities of vitamin-deficient suspensions of *S. aureus*

Addition to the vitamin-deficient *S. aureus*	Specific activities of enzymes, nmoles/mg protein/min				
	Glucose-6-P dehydrogenase	6-P-gluconate dehydrogenase	Isocitrate dehydrogenase	NADH oxidase	NADPH oxidase
None	42	12	12	6	3
Niacin (2 μg/ml) + thiamine (2 μg/ml)	43 (0.19[a])	20 (0.8)	15 (0.3)	8 (0.3)	5 (0.47)
Niacin + thiamine + chloramphenicol (50)	44 (0.32)	13 (0.1)	16 (0.57)	9 (0.5)	4 (0.34)
Chloramphenicol (50)	40 (0.6)	20 (0.8)	17 (0.7)	10 (0.56)	5 (0.69)

Cells were grown in 2% Vitamin-free Casitone for 17 hr, washed with, and suspended in 0.05 M Na and K phosphate buffer, pH 7.0, at twice their original concentration. Glucose was added at a concentration of 50 μmoles/ml and after the indicated additions were made the flasks were placed on a rotary shaker at 37 C for 3 hr. The cells were then washed and the enzyme activities were determined in the crude extracts by the method of Ragland et al. (96).

[a] Difference of means by "t" test; a value of 2.0 or more is significant at the 5% level or better.

(5) Changes in glucose catabolic pathways and in enzyme levels by the addition of vitamins in the absence of protein synthesis. To insure that protein synthesis was not proceeding from the endogenous amino acid pool, vitamin-deficient staphylococcal cells were incubated in a buffered glucose medium containing niacin and thiamine (2 μg/ml, each), and along with any of the following antibiotics: puromycin, chloramphenicol, or actinomycin D.

The results of the experiments, presented in Table 6, were essentially the same as those reported in Table 4, in that niacin and thiamine stimulated the HMP pathway and the TCA cycle activity (ca. 18% and 4%) even in the presence of antibiotics. The chloramphenicol control experiment (Table 4, Expt. 4) in which the vitamins were omitted showed that the antibiotic itself was not responsible for any of the observed biochemical changes. In the presence of actinomycin D, over 30% of the glucose was catabolized via the HMP pathway, following the addition of vitamins, a value considerably higher than that reached in the absence of the antibiotic

Analyses of enzyme activities again demonstrated that (following the addition of the vitamins), the level of G6PD was not significantly increased over the control value (44 units, Table 5). The specific activities of the other enzymes also remained less and variable.

TABLE 6

Effect of niacin and thiamine on some biochemical properties of vitamin-deficient *S. aureus* in the absence of protein synthesis

	Vitamin-deficient resting cells[a] incubated in buffered glucose containing niacin + thiamine (5 μg/ml) and:					Resting cells[b] previously grown on Vitamin-supplemented (5 μg/ml niacin + thiamine) medium
	No antibiotic		Chloramphenicol, 25 μg/ml	Puromycin, 6 μg/ml	Actinomycin D, 3 μg/ml	
	0 Hr	3 Hr	3 Hr	3 Hr	3 Hr	
Intact Cells						
% Glucose via HMP	5.0	16.0	14.0	18.5	34.0	16.0
Rel. act. of TCA	0.6	4.0	4.0	5.7	5.0	4.0
Cell Extracts						
NAD, μmoles/g. dry wt.	0.2	3.5	3.8	3.5	13.2	6.7
NADP, μmoles/g/ dry wt.	0.2	0.4	0.4	0.5	2.2	0.5
Glucose-6-P dehydrogenase, Sp. Act.	46.0	42.0	42.0	-	-	145.0
Isocitrate dehydrogenase, Sp. Act.	22.0	13.0	16.0	-	-	58.0

a Cells were grown for 17 hr in unsupplemented 2% Vitamin-free Casitone, harvested, washed with saline, and suspended in 0.05 M phosphate buffer, pH 7.0, containing 0.9% glucose, niacin and thiamine.

b Cells were grown for 17 hr in 2% Vitamin-free Casitone containing niacin and thiamine, and suspended in 0.05 M phosphate buffer, pH 7.0, with no vitamins.

Since the addition of the vitamins under resting cell conditions did not significantly increase the specific activities of the enzymes tested, and since the use of antibiotics precluded protein synthesis, new enzyme synthesis as a possible explanation for the increased activity of the HMP pathway has been eliminated in the present studies.

Parallel experiments in this laboratory have demonstrated that when the vitamin-deficient resting cells were incubated with antibiotics for 3 hr with either niacin or niacin and thiamine, the NAD and NADP levels increased 10- and 2-fold, respectively (44).

(6) The effect of starvation on glucose catabolic pathways of *S. aureus*. Results of the previous experiments (Table 4) indicate that increased enzyme levels were not responsible for the increased operation of the HMP pathway following addition of niacin. Since there were 10- and 2-fold increases in the NAD and NADP levels, respectively, along with increased activity of the HMP cycle, it is likely that one of the coenzymes regulated the HMP pathway activity of *S. aureus* in some manner. Since the NAD content in *E. coli* has been found to decrease during a period of incubation in buffered glucose (86), experiments were therefore designed to determine whether such a variation in NAD could be observed in staphylococci during starvation and, if so, to correlate the NAD and NADP levels with the

degree of utilization of the EM and HMP pathways.

Fig. 3 presents the results of the glucose catabolic pathways during starvation of staphylococcal cells previously grown in Vitamin-free Casitone supplemented with 0.5 μg/ml of niacin and 2.0 μg/ml of thiamine. This niacin level was selected to yield cells with a high level of glucose oxidation via the HMP pathway, but with levels of NAD that were not high. The results of nicotinamide coenzymes are included for the purpose of comparison (Fig. 3).

During the 2 hr period of starvation the operation of the HMP pathway was reduced from 18.5% to 10%. Similarly, a 50% reduction in NAD content was observed (i.e., 2.3 to 1.2 micromoles/g dry weight). Throughout the incubation period the level of NADP was not reduced and remained over 0.4 micromoles/g. There was no further decline in the HMP pathway as the incubation continued. The amount of glucose catabolized via the HMP cycle was approximately 9.8% after 4 and 6 hr of starvation, compared to 18.5% before starvation. Further reduction in NAD content from 1.0 to 0.6 micromoles/g was noted, and this level remained unchanged as the incubation continued. The level of NADP did not decrease but rather increased as the incubation period continued up to 6 hr. Throughout the period of starvation the activity of TCA cycle decreased slightly but was still quite active. Thus, the TCA cycle activity at 0, 2, 4 and 6 hr was 4.5,

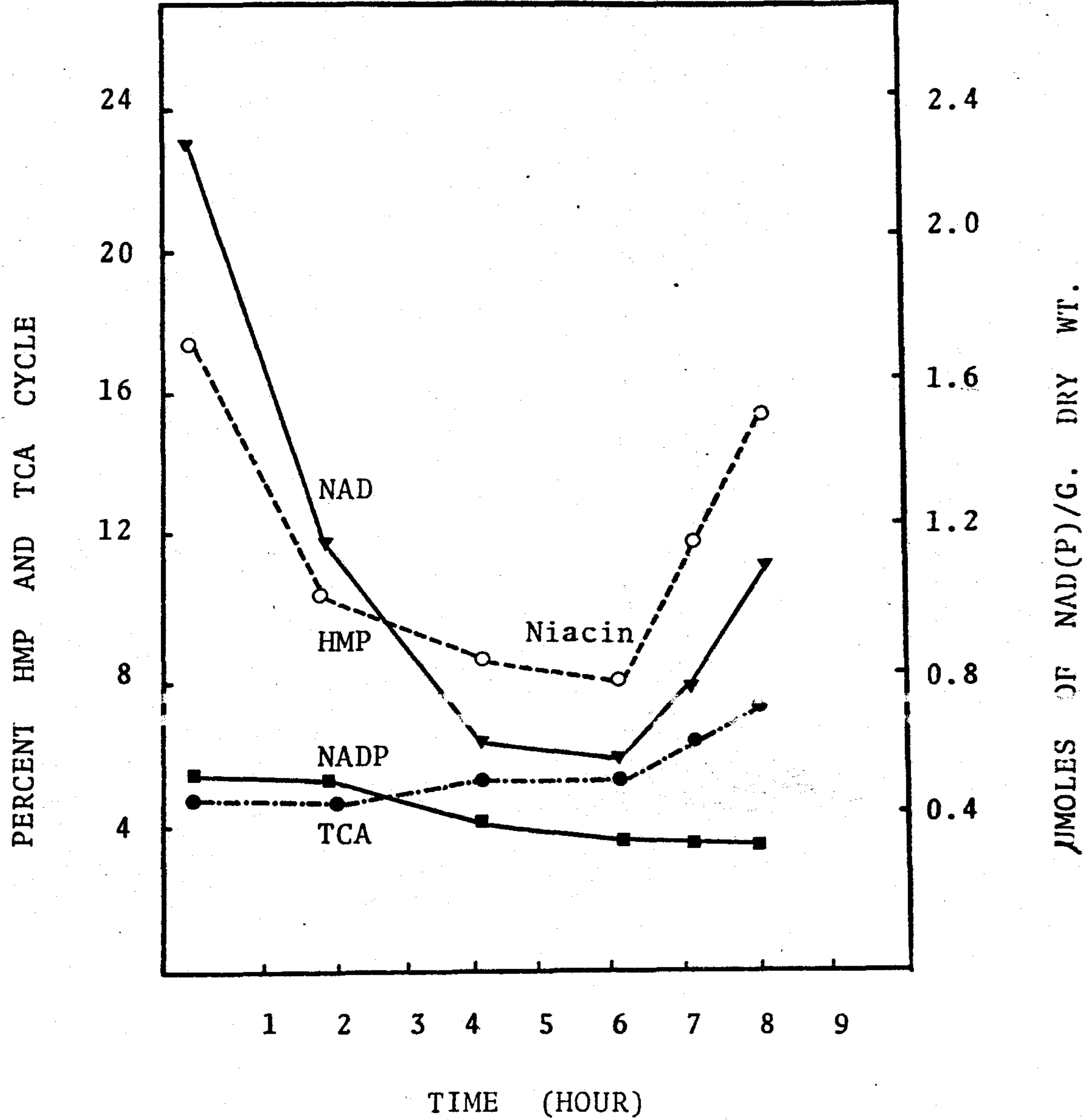

Figure 3. Biochemical changes in S. aureus during incubation in buffered glucose. Cells were grown for 17 hr in 2% Vitamin-free Casitone supplemented with 0.25 µg/ml niacin and 4 µg/ml thiamine, washed with saline and incubated in 0.05 M K phosphate buffer, pH 7.0, containing glucose. At intervals samples were removed for the analyses of glucose catabolic pathways in a manner previously described (see Methods).

4.0, 3.8 and 3.0 percents, respectively.

At the end of the 6 hr incubation period niacin (100 μg/ml) was added to the buffered glucose medium and the incubation was continued for another 2 hr. Following this procedure, the operation of the HMP pathway increased from 10% to 19% while the NAD content was increased to 1.3 μmoles/g dry weight. The NADP content also rose from 0.6 to 0.7 μmoles/g. On the basis of these findings it is evident that for staphylococci, NAD levels above (approximately) 1.5 μmoles/g were necessary for the operation of the HMP pathway at levels above 20%.

C. Studies with niacin analogs

(1) Growth curve of S. aureus in Vitamin-free Casitone in the presence of niacin analogs. The results of preliminary studies on the effect of several niacin analogs on the growth of S. aureus, Towler, in Vitamin-free Casitone are presented in Fig. 4 and 5. In Vitamin-free Casitone (Fig. 4) the small amount of growth (turbidity of 70 Klett units) tripled upon the addition of niacin. When niacin was replaced by niacin analogs in concentrations ranging from 100 to 1000 μg/ml, there was a moderate increase in growth. For instance, none of the analogs produced turbidity readings over 150 Klett units in 24 hr of incubation in comparison to the 250 Klett units produced in the presence of niacin. In the presence of thiamine (Fig. 5), however,

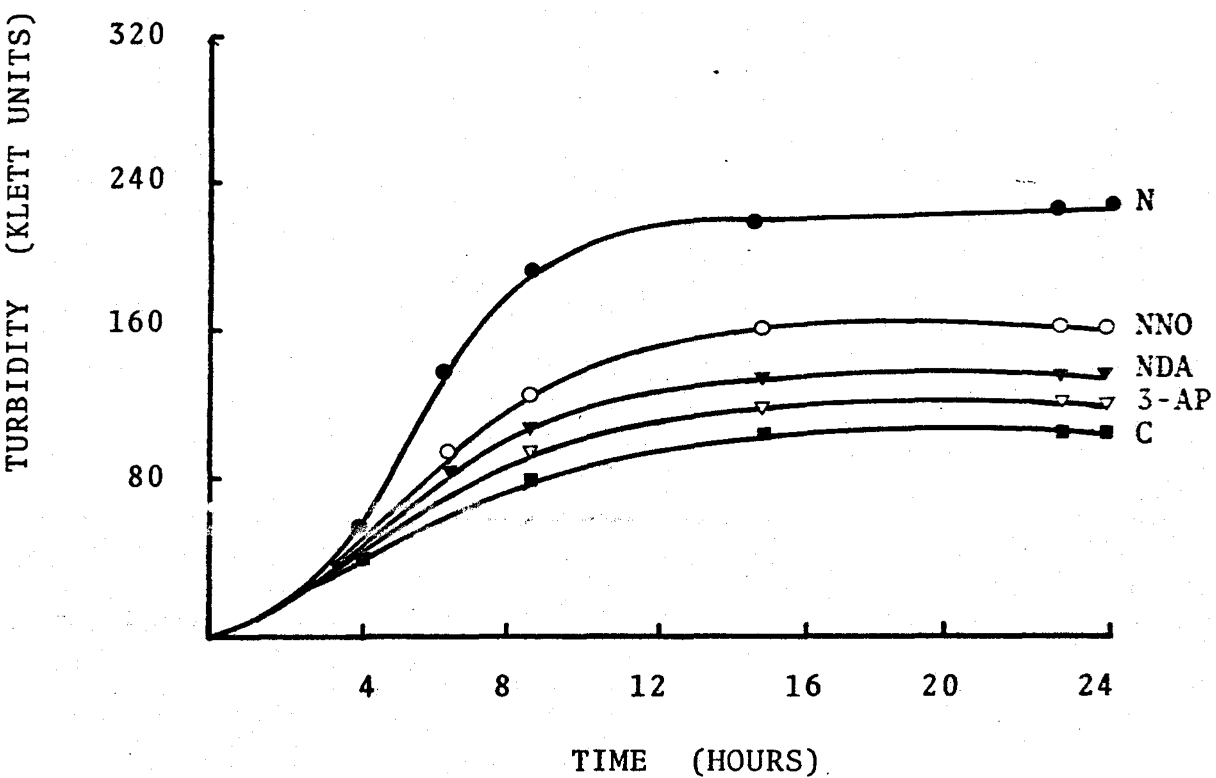

Figure 4. <u>Growth of S. aureus in 2% Vitamin-free Casitone with added analogs of niacin</u>. Experimental conditions were same as in Fig.1. N=niacin, 2 μg/ml; NNO=nicotinamide-N-oxide, 100 μg/ml; NDA=nicotinyldiethylamide, 100 μg/ml; 3-AP=3-acetylpyridine, 1000 μg/ml; C=unsupplemented Vitamin-free Casitone.

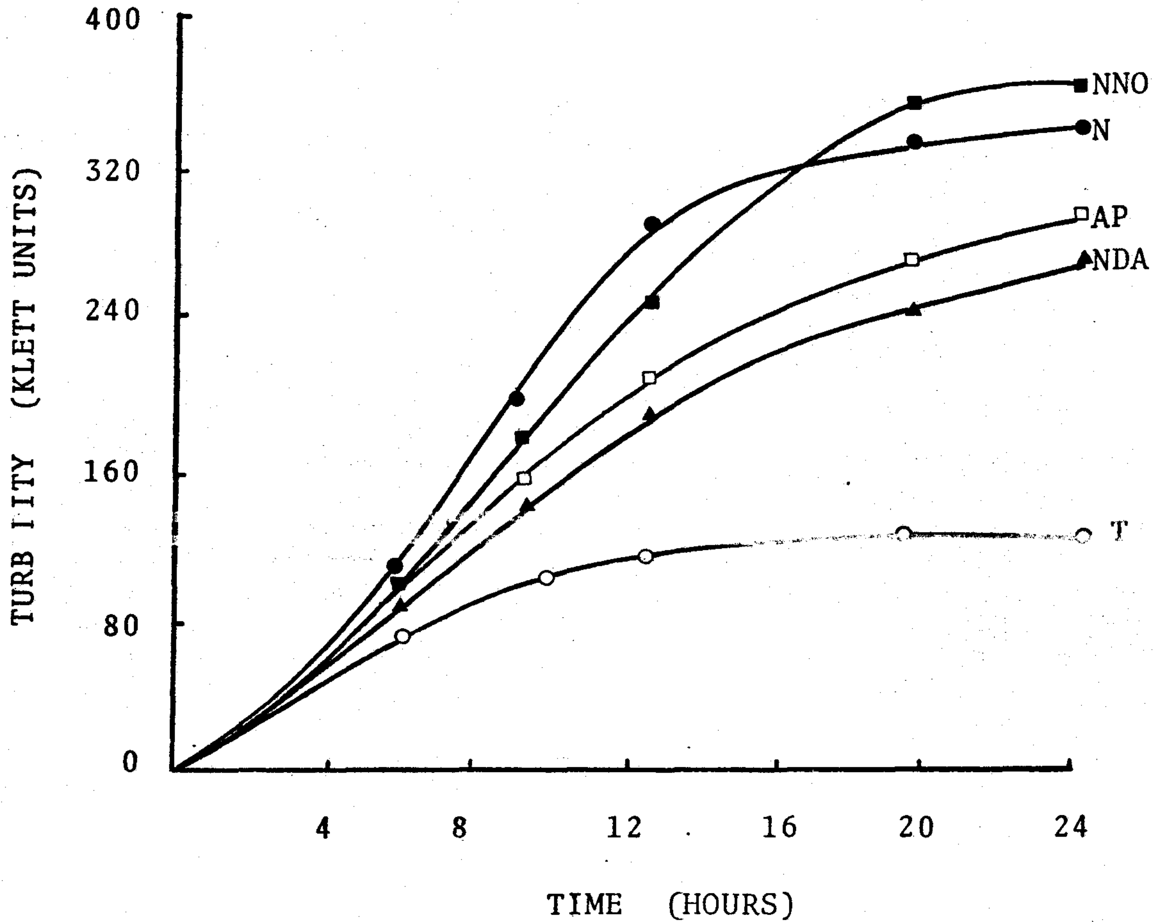

Figure 5. <u>Growth of S. aureus in 2% Vitamin-free Casitone with added niacin analogs and thiamine</u>. Experimental conditions were same as in Fig.1. The concentrations of niacin, thiamine and of niacin analogs were same as in Fig. 4.

all the analogs tested with the exception of 6-HN and 6-AN produced approximately the same stimulation as the addition of niacin and thiamine. For example, when nicotinamide-N-oxide (NNO), thionicotinamide (ThN), or 3-acetylpyridine (3AP) were added to a thiamine supplemented growth medium, the turbidity measurement reached a maximum of 330 to 360 Klett units.

Data from Fig. 4 and 5 indicated that the concentration of niacin analogs required for the maximal stimulation of growth was approximately 100- to 1000-fold higher than that of niacin. The use of 3-acetylpyridine or pyridine-3-sulfonic acid required 1000 μg/ml to yield maximal cell growth (Fig.5 and Table 13). Necessary concentrations of other pyridine derivatives varied somewhat from 100 to 500 μg/ml in order to support full staphylococcal growth when added to the Vitamin-free Casitone medium with added thiamine.

In addition, results of these growth curve studies demonstrated no lag in the growth in the presence of niacin analogs. The duration of the observed lag period was similar to that produced when niacin was added to the growth medium. This indicated that the growth-stimulating effect of the addition of analogs was not due to selection of mutants capable of using analogs.

(2) Growth of S. aureus in synthetic medium in the presence of niacin analogs. Since Vitamin-free Casitone contains small and variable amounts of niacin and thiamine, the effect of the niacin analogs on *S. aureus* growth in completely synthetic medium was investigated. The results of these studies are presented in Table 7. The findings of these experiments showed practically no growth of staphylococci in the absence of either niacin or niacin and thiamine. A turbidity reading of 55 Klett units was obtained when niacin alone was present in the synthetic medium, while growth reached a turbidity of 270 Klett units (48 hr) when both vitamins were present in the medium. In the presence of niacin analogs in the medium lacking thiamine, growth varied somewhat from 11 to 40 Klett units. Data in Table 7 shows that, of all pyridine derivatives tested, nicotinamide-N-oxide, thionicotinamide and nicotinuric acid yielded maximal growth stimulation (i.e., 270 to 343 Klett units) in synthetic medium with added thiamine. There was very little growth when 6-hydroxynicotinic acid or 6-aminonicotinamide was added to a thiamine-supplemented synthetic medium. Higher yield of cells obtained upon addition of nicotinamide-N-oxide, thionicotinamide or pyridine-3-aldehyde to the growth medium indicates that these analogs could be used more effectively than niacin in stimulating growth of staphylococci.

TABLE 7

Effect of niacin analogs on the growth of *S. aureus* in a synthetic medium

Compounds tested	Concn μg/ml	Synthetic medium		Synthetic medium + thiamine	
		Turbidity[a] in Klett units, after			
		24 hr	48 hr	24 hr	48 hr
None	-	2	6	5	15
Niacin	8	55	54	239	270
Nicotinamide-N-oxide	100	16	28	293	343
Thionicotinamide	500	15	28	254	315
Nicotinuric acid	8	9	28	226	271
3-Acetylpyridine	1000	5	11	134	205
Nicotinyldiethylamide	100	13	24	107	150
Pyridine-3-sulfonic acid	250	26	40	195	110
6-Hydroxynicotinic acid	500	2	6	7	15
6-Aminonicotinamide	100	3	3	4	10

a Turbidity measurements were made at 600 nm.

(3) Effect of niacin analogs on growth of different strains of staphylococci and Gram negative bacteria in synthetic medium supplemented with thiamine. To insure that niacin analogs replaced niacin in more than one strain of *S. aureus* additional strains of *S. aureus*, *S. epidermidis* and a few niacin requiring Gram negative bacteria were tested. The results of these growth studies are shown in Table 8. The data indicate that 3-acetylpyridine and nicotinyldiethylamide could reproduce the effect of niacin on the growth of these organisms. Although the response of the organisms varied somewhat, growth stimulation was noted in all the cases. Results from Table 8 show that when 3-acetylpyridine (1000 μg/ml) or nicotinyldiethylamide (100 μg/ml) was added to a thiamine-supplemented synthetic medium, the growth of tested strains of *S. aureus* and *S. epidermidis* was stimulated (over 150 Klett units), and the cell yields were similar to those obtained following the addition of niacin and thiamine to the synthetic medium. The data also indicates that niacin-requiring *S. dysenteriae*, *P. vulgaris* and *P. morgani* successfully used either 3-acetylpyridine or nicotinyldiethylamide for growth stimulation.

(4) Effect of niacin analogs on the glucose catabolic pathways of S. aureus. Preliminary experiments (Table 1) had shown that addition of niacin and thiamine to

TABLE 8

Growth of staphylococci and of Gram negative bacteria in a thiamine-supplemented synthetic medium with niacin or niacin analogs

Strain	Niacin or niacin analogs added to the synthetic medium					
	Niacin, 8 μg/ml		3-Acetylpyridine, 1000 μg/ml		Nicotinyldiethylamide, 100 μg/ml	
	Turbidity in Klett units after 24 and 48 hr					
S. aureus	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
Towler	239	270	134	205	107	150
3	92	185	171	168	30	170
10	229	264	184	176	89	93
18Z	228	270	166	228	41	168
Serotype I	276	274	189	183	131	173
Serotype III	242	282	192	257	221	271
Serotype IV	155	264	215	224	174	225
S. epidermidis						
155	117	228	110	234	44	160
12228	212	212	189	199	63	155
14990	220	215	192	272	172	180
Shigella flexneri	145	137	122	117	103	115
Proteus morgani	210	200	202	209	183	159
Proteus vulgaris	221	195	175	182	186	167

Synthetic medium was devoid of nicotinic acid.
Turbidity measurements were made at 600 nm; the values are the average of two separate experiments.

the growth medium had considerable influence on the metabolic activities of *S. aureus*. Therefore, the effect of niacin analogs on glucose catabolic pathways of staphylococcal cells was investigated. The results of measurements of the glycolytic pathways of *S. aureus* grown in 2% Vitamin-free Casitone with added niacin analogs and thiamine are shown in Table 9. Those compounds which were highly active are included in this table. Approximately 18% of the glucose was metabolized via the HMP pathway when niacin and thiamine were added to the medium. All of the analogs listed in Table 9, except 6-hydroxynicotinic acid and 6-aminonicotinamide, produced similar increases in the HMP pathway utilization and in the TCA cycle activity. For instance, when niacin analogs and thiamine were added to the growth medium, the amount of glucose catabolized via the HMP pathway ranged from 15 to 24%, and the relative activity of the TCA cycle was 4.5 to 7.0%, as compared to 4.5 when niacin and thiamine were added to the growth medium.

The data also show that the concentrations of the analogs required to stimulate the HMP pathway were 100- to 1000-fold higher than that of niacin. As shown in Table 9, when nicotinamide-N-oxide (100 μg/ml) and 3-acetylpyridine (1000 μg/ml) were added to the growth medium, about 15% and 18.7% of the glucose was oxidized via the HMP pathway,

TABLE 9

Effect of niacin analogs on the glucose catabolic pathways and on coenzyme levels of *S. aureus*

Addition (μg/ml) to Vit.-free Casitone	% Glucose via HMP	Rel. Act. of TCA (%)	Concn of[a]	
			NAD	NADP
None	5.0	0.2	0.3	0.2
Thiamine (4)	5.2	1.2	0.4	0.2
Niacin (8)	22.0	4.5	4.8	0.6
Niacin (8) + thiamine (4)	18.0	4.5	3.5	0.4
Nicotinuric acid (8) + thiamine (4)	19.9	6.1	2.7	0.4
Nicotinamide-N-oxide (100) + thiamine (4)	15.1	7.4	3.0	0.4
3-Acetylpyridine (1000) + thiamine (4)	18.7	5.2	0.4	0.1
Nicotinyldiethylamide (100) + thiamine (4)	18.3	5.1	1.0	0.2
6-Hydroxynicotinic acid (500) + thiamine (4)	7.8	3.7	0.4	0.3
3-Pyridylcarbinol (100) + thiamine (4)	24.2	4.6	3.2	0.6

% Glucose catabolized via the EM and the HMP pathway and the relative activity of TCA cycle were estimated as described previously (see Methods).

[a] Nicotinamide coenzymes values are expressed in μmoles/g dry weight.

respectively.

These readings indicate that niacin analogs could replace niacin for stimulation of glucose utilization via the HMP pathway in *S. aureus*. The results of these experiments also indicate that addition of 6-hydroxynicotinic acid to the growth medium did not increase the activity of the HMP pathway, although it caused significant changes in the TCA cycle activity. Due to these unexpected results 6-hydroxynicotinic acid was further investigated; the results of the experiments are presented in Table 15 of this section.

Since earlier experiments suggested that some of the niacin analogs could completely replace niacin for stimulation of staphylococcal growth and could also increase glucose utilization via the HMP pathway, additional pyridine derivatives were investigated. On the basis of the results on growth and on the HMP pathway utilization of *S. aureus*, the niacin analogs have been classified into 4 groups: the results are presented in Table 10 with the chemical structure of all the compounds shown.

D. Classification of niacin analogs

Group I. Analogs stimulating growth and the HMP pathway maximally. In this category 25 analogs that maximally stimulated HMP pathway utilization and growth in 2% Vitamin-free Casitone suppliemented with thiamine are

included. For instance, growth reached a turbidity of 340 to 360 Klett units while the amount of glucose oxidized via the HMP pathway was 15 to 23%.

Group II. Analogs allowing partial stimulation of growth and of HMP pathway activity. In this category the following analogs are included: 3-pyridylacetic acid, 3-ethylpyridine, 6-chloronicotinamide, dipicolinic acid, picolinic acid, 2-aminonicotinic acid, pyridine-2 and -4-aldehyde and 2-chloronicotinic acid. It can be seen that most of these analogs were substituted at the 2-, 4- and 6-positions of the pyridine ring. All of these pyridine derivatives partially stimulated growth and HMP pathway activity. For example, growth did not exceed a turbidity of 160 to 180 Klett units, and the amount of glucose utilized via the HMP pathway ranged from 10 to 14% in the cells grown in a thiamine supplemented Vitamin-free Casitone. Most of the analogs were tested at 3 or 4 different concentrations. Even when the concentration of these analogs was increaed to 5000 μg/ml, there was neither increase in the amount of growth nor in the utilization of the HMP pathway.

Group III. Analogs having no effect on either growth or on the HMP pathway. This category included 4-acetylpyidine, 3-benzoylpyridine, pyridine, pyridoxine, 2,6-diacetylpyridine, 3,4-diaminopyridine, 2-picoline,

4-picoline, 3-hydroxypyridine-N-oxide and other pyridine derivatives (Table 10). The analogs were tested using a wide range of concentrations and the results showed that the addition of these analogs to a thiamine-supplemented Vitamin-free Casitone did not increase the growth over the control level (70 Klett units). Similarly, increase in glucose oxidation via the HMP pathway was not observed. For instance, staphylococcal growth reached a turbidity of only 60 to 70 Klett units, the same as unsupplemented media, and the level of HMP pathway activity remained at 6-8%.

Group IV. Analogs with complete inhibition of growth. This category included 6-aminonicotinamide, 3-hydroxypicolinic acid, 5-fluoronicotinamide and pyrizinamide. The addition of these analogs to the growth medium resulted in complete inhibition of staphylococcal growth.

E. Correlation between growth and the glycolytic pathways.

(1) Influence of niacin and niacin analog concentrations on growth and on the glycolytic pathways of S. aureus. The results of a number of experiments have suggested that growth stimulation and stimulation of the HMP pathway activity are independent of each other. This was first clearly demonstrated in studies with

TABLE 10

The ability of various pyridine derivatives to replace niacin for staphylococcal growth and for the stimulation of the HMP pathway activity and NAD concentration.

Compound	Structure	Stimulation of		
		Growth	HMP activity	NAD concn
Group I				
Nicotinic acid (niacin)	-COOH, N	+ +	+ +	+ +
Nicotinamide (niacinamide)	-C(=O)-NH_2, N	+ +	+ +	+ +
Nicotinic acid-N-oxide	-COOH, N→O	+ +	+ +	+ +
Ethylnicotinate	-C(=O)-C_2H_5, N	+ +	+ +	+ +
Isoniazid (isonicotinyl-hydrazide)	O=C-NH-NH_2, N	+ +	+ +	+ +
Isonicotinic acid	COOH, N	+ +	+ +	+ +

Table 10 (Continued)

Compound	Structure	Stimulation of		
		Growth	HMP activity	NAD concn
Isonicotinamide	O=C-NH_2, N	+ +	+ +	+ +
Nicotinamide-N-oxide	O, -C-NH_2, N→O	+ +	+ +	+ +
N-Methylnicotinamide	O, -C-NH_2, N-CH_3	+ +	+ +	+ +
N-Ethylnicotinamide	O, -C-NH_2, N-C_2H_5	+ +	+ +	+ +
Thionicotinamide	S, -C-NH_2, N	+ +	+ +	+ +
Nicotinuric acid (nicotinyl glycine)	O, -C-N(H)-CH_2COOH, N	+ +	+ +	+ +

Table 10 (Continued)

Compound	Structure	Stimulation of		
		Growth	HMP activity	NAD concn
Nicotinyldiethyl-amide (coramine)	$-C-N(CH_2CH_3)(CH_2CH_3)$	+ +	+ +	+ +
Pyridine-3-sulfonic acid	$-S(O)(O)-OH$	+ +	+ +	+ +
2-Picoline-N-oxide	$-CH_3$	+ +	[illegible]	[illegible]
3-Picoline-N-oxide	$-CH_3$	+ +	+ +	+ +
Pyridine-N-oxide		+ +	+ +	+ +
3-Acetylpyridine	$-C(O)-CH_3$	+ +	+ +	+ +

Table 10 (Continued)

Compound	Structure	Stimulation of Growth	Stimulation of HMP activity	Stimulation of NAD concn
Pyridine-3-aldehyde	pyridine ring with -C(=O)-H at position 3	+ +	+ +	+ +
4-Pyridylcarbinol	pyridine ring with CH_2OH at position 4	+ +	+ +	+ +
3-Pyridylcarbinol	pyridine ring with CH_2OH at position 3	+ +	+ +	+ +
3-Pyridylcarbinol-N-oxide	pyridine ring with CH_2OH at position 3, N→O	+ +	+ +	+ +
Quinolinic acid	pyridine ring with COOH at positions 2 and 3	+ +	+ +	+
5-Chloronicotinic acid	Cl- pyridine ring -COOH	+ +	+ +	- +

Table 10 (Continued)

Compound	Structure	Stimulation of Growth	HMP activity	NAD concn
Group II				
2-Chloronicotinic acid	Pyridine ring with -COOH and -Cl	+	+	-
2-Aminonicotinic acid	Pyridine ring with -COOH and $-NH_2$	+	+	ND
3-Pyridylacetic acid	Pyridine ring with $-CH_2COOH$	+	+	+
Picolinic acid	Pyridine ring with -COOH	+	+	+
Dipicolinic acid	HOOC- pyridine ring -COOH	+	+	+
Pyridine-2-aldehyde	Pyridine ring with -C(=O)-H	+	+	+

Table 10 (Continued)

Compound	Structure	Stimulation of		
		Growth	HMP activity	NAD concn
Pyridine-4-aldehyde	O=C-H (pyridine ring, N)	+	+	-
6-Chloronicotinamide	Cl- (pyridine ring, N) -C(=O)-NH_2	+	+	-
3-Ethylpyridine	(pyridine ring, N) -CH_2CH_3	+	+	
Group III				
3-Benzoylpyridine	(pyridine ring, N) -C(=O)- (benzene ring)	-	-	ND
3,4-Diaminopyridine	NH_2, -NH_2 (pyridine ring, N)	-	-	ND
3-Hydroxypyridine-N-oxide	-OH (pyridine ring, N-O)	-	-	-

Table 10 (Continued)

Compound	Structure	Stimulation of Growth	Stimulation of HMP activity	Stimulation of NAD concn
2,4-Pyridinedicarboxylic acid	COOH, -COOH, N	-	-	ND
Pyridine	N	-	-	-
4-Acetylpyridine	$O{=}C{-}CH_3$, N	-	-	ND
Pyridoxine	CH_2OH, HO-, $-CH_2OH$, H_3C-, N	-	-	ND
6-Hydroxynicotinic acid	-COOH, HO-, N	-	-	-
5-Fluoronicotinic acid	F-, -COOH, N	-	-	-

Table 10 (Continued)

Compound	Structure	Stimulation of		
		Growth	HMP activity	NAD concn
2-Hydroxynicotinic acid	COOH, OH, N	-	-	ND
4-Hydroxynicotinic acid	OH, COOH, N	-	-	ND
2-Picoline	CH_3, N	-	-	-
4-Picoline	CH_3, N	-	-	-
<u>Group IV</u>				
6-Aminonicotinamide	$C(=O)-NH_2$, H_2N-, N	i	i	-
3-Hydroxypicolinic acid	OH, COOH, N	i	ND	ND

Table 10 (Continued)

Compound	Structure	Stimulation of		
		Growth	HMP activity	NAD concn
5-Fluoronicotinamide	F–(pyridine ring)–C(=O)–NH_2	i	ND	ND
Pyrizinamide	(pyrazine ring)–C(=O)–NH_2	i	ND	ND

Symbols:
+ +, Maximal stimulation
+, Partial stimulation
−, No stimulation
i, Inhibition
ND, Not determined

3-pyridylcarbinol (Table 11). There appeared to be two distinct effects of 3-pyridylcarbinol (3PC) which are concentration-dependent. At a concentration of 2 μg/ml growth was stimulated submaximally (147 Klett units) while at a concentration of 10 μg/ml of 3PC turbidity measurements reached 355 Klett units, indicating that maximum growth had been reached. Yet analyses of the glucose catabolic pathways by cells grown with different levels of 3PC (2 and 10 μg/ml) in the medium showed that only 7 to 9% of the glucose was catabolized via the HMP pathway with partial stimulation of the TCA cycle activity (2.2%). Thus, results from Table 11 indicate that concentrations of 10 and 50 μg/ml were sufficient to produce maximum growth but not sufficient to stimulate the glycolytic pathways. A concentration of 100 μg/ml or more of 3PC, however, was shown to increase both growth and the utilization of the HMP pathway maximally in staphylococci. It can be seen that along with the maximum growth the amount of glucose oxidized via the HMP pathway and the relative activity of the TCA cycle were 27.0% and 3.7%, repsectively. When a concentration of 5000 μg/ml of 3PC was present in the growth medium the amount of growth (320 Klett units) and the activity of the HMP pathway (22.1%) remained at maximal levels, but a reduction in

TABLE 11

Effect of the addition of different concentrations of 3-pyridylcarbinol to a thiamine-supplemented Vitamin-free Casitone medium on growth and on glucose catabolism of *S. aureus*

Concentration µg/ml	Turbidity,[b] Klett units	TCA cycle activity (%)	% Glucose[a] catabolized via EM	HMP
2	147	2.2	92.3	7.7
10	355	2.2	90.9	9.0
50	338	3.0	82.5	17.5
100	340	3.7	72.9	27.0
500	325	4.7	74.9	25.1
1000	300	3.2	77.2	22.8
5000	320	1.5	77.8	22.1

a Glucose catabolic pathways participation and the TCA cycle activity were estimated as described previously (see methods).

b Growth was determined by measuring the turbidity at 600 nm after 17 hr.

The values are the average of two separate experiments.

the TCA cycle activity from 4.7% to 1.5% was noted.

Since such separate effects on growth and on the HMP pathway were observed depending on the concentration of the analog used in the growth medium, studies were conducted with various concentrations of niacin and the results are shown in Table 12. The results indicated that 0.05 μg/ml of niacin in thiamine-supplemented Vitamin-free Casitone produced almost maximal growth (300 Klett units) without stimulating the utilization of the HMP pathway for glucose oxidation. Only 9-10% of the glucose was oxidized via the HMP pathway. As the concentration of niacin was increased from 0.05 to 0.25 μg/ml, a gradual increase in the activity of the HMP pathway was noted. For example, maximum utilization of glucose via the HMP pathway (21.6%) occurred at a concentration of 0.25 μg/ml of niacin. When the niacin concentration in the medium was increased to 1000 μg/ml, there was a reduction in the growth rate as well as in the operation of the HMP pathway. For instance, decline in the turbidity readings of 162 Klett units was observed, while the relative activities of HMP and the TCA cycles were 13.4% and 3.3%, respectively. With the addition of 2000 μg/ml there was complete inhibition of staphylococcal growth.

TABLE 12

Effect of the addition of different concentrations of niacin to a thiamine-supplemented Vitamin-free Casitone on growth and on glucose catabolism of *S. aureus*.

Niacin added µg/ml	Turbidity,[b] Klett units	TCA cycle activity (%)	% Glucose[a] catabolized via EM	HMP
0.00	42	0.7	90.0	9.1
0.05	300	2.0	89.2	10.8
0.10	326	3.6	84.5	13.5
0.25	335	5.6	78.4	21.6
0.50	315	2.5	79.4	20.5
2.00	345	4.5	82.0	18.0
5.00	341	2.9	82.5	17.5
50.00	335	4.3	80.3	19.6
500.00	330	3.9	75.6	24.4
1000.00	162	3.3	86.6	13.4
2000.00	11	-	-	-

a Glucose catabolic pathway participation and TCA cycle activity were estimated as described previously (see Methods).

b Growth was determined by measuring the turbidity at 600 nm after 17 hr.

The values are the average of two separate experiments.

The niacin content of 2% Vitamin-free Casitone is 0.0013 µg/ml.

TABLE 13

Effect of the addition of different concentrations of pyridine-3-sulfonic acid to a thiamine-supplemented Vitamin-free Casitone medium on growth and on glucose catabolism of *S. aureus*

Concentration µg/ml	Turbidity,[b] Klett units	TCA cycle activity (%)	% Glucose[a] catabolized via	
			EM	HMP
250	201	1.7	89.3	10.7
500	272	1.2	92.0	8.0
1,000	380	1.4	92.7	7.3
2,000	405	3.0	85.8	14.2
5,000	345	3.5	80.6	19.4
10,000	430	3.5	68.4	31.6
20,000	425	3.2	70.0	30.0

[a] Glucose catabolic pathway participation and TCA cycle activity were estimated as described previously (see Methods).

[b] Growth was determined by measuring the turbidity at 600 nm after 17 hr.

The values are the average of two separate experiments.

These results with niacin substantiated the present study's previous observation in the case of 3PC where low concentration of the vitamin was required for maximum stimulation of growth and higher concentration was needed for maximum operation of the glycolytic pathways.

Table 13 showed similar results; different concentrations of pyridine-3-sulfonic acid in Vitamin-free Casitone were used with added thiamine. Essentially similar results were obtained as those reported with niacin and 3-pyridylcarbinol (Tables 11 and 12), except that there was a wider margin between the concentrations required for maximal growth and for maximal HMP stimulation.

F. Effect of niacin analogs on the glycolytic pathways.

(1) Effect of 6-aminonicotinamide on the HMP pathway of *S. aureus*. The results with the 6-substituted analog of niacin, 6-aminonicotinamide (6-AN), on the growth and on the activity of the HMP pathway are shown in Table 14. When 100 μg/ml of this analog of niacin was added to Vitamin-free Casitone, it completely inhibited growth of *S. aureus*. The turbidity of <25 Klett units obtained after incubation for 24 to 48 hr was probably due to traces of vitamins present in the growth medium. There was no further increase in the growth when the incubation was continued for as long as 72 hr (results

TABLE 14

Effect of 6-aminonicotinamide (6-AN) on the glucose catabolic pathways of *S. aureus*

Addition to the 2% Vitamin-free Casitone growth medium	Additions to the resting cells grown in column 1 medium	Incubation time hr	TCA cycle activity (%)	% Glucose catabolized via	
				HMP	EM
None	None	3	0.4	8.3	91.7
None	6-AN (100 μg/ml) + thiamine (2 μg/ml)	3	0.2	2.4	97.6
Niacin (2 μg/ml) + thiamine (2 μg/ml)	6-AN (100 μg/ml) + thiamine (2 μg/ml)	3	4.5	20.3	79.7
Niacin (2 μg/ml) + thiamine (2 μg/ml)	6-AN (100 μg/ml) + thiamine (2 μg/ml)	6	2.3	8.9	91.9
Niacin (0.05 μg/ml) + thiamine (2 μg/ml)	None	2	2.4	9.0	91.0
Niacin (0.05 μg/ml) + thiamine (2 μg/ml) + 6-AN (100 μg/ml)	None	2	2.0	0.3	99.7

The cells were grown for 17 hr under various conditions as indicated in the 1st column. After growth they were washed with and suspended in 0.05 M phosphate buffer with added niacin, thiamine or 6-AN as shown in the 2nd column. The flasks were placed on a rotary shaker for the indicated time. These cells were then harvested and used in the usual manner as described previously (see Methods).

not shown in Table 14). Due to the lack of growth in medium with added 6-AN, analyses of the glucose catabolic pathways were not performed.

It was possible to study the effect of 6-AN on the glycolytic pathways under resting cell conditions. When staphylococci that had previously grown in unsupplemented Vitamin-free Casitone were washed and incubated in phosphate buffer (0.05 M, pH 7.0)-glucose (0.9%) medium containing 6-AN(100 μg/ml) and thiamine (2 μg/ml) for 2-3 hr, there was a reduction in the utilization of the HMP pathway from 8.3% to 2.4% (Table 14). Decrease in the activity of the HMP pathway resulting from the addition of 6-amino-nicotinamide was also observed in niacin grown cells which utilized HMP pathway maximally. For instance, when staphylococcal cells were first grown in supplemented Vitamin-free Casitone and then incubated in buffered glucose with 6-AN and thiamine, the activity of the HMP pathway was unaffected for the first 3 hr. As the incubation continued for 6 hr, a marked decrease in the utilization of the HMP pathway (from 20.3% to 8.9%) was noted. This strongly indicates that 6-AN in some manner inhibits the activity of the HMP pathway in *S. aureus*.

The results of the experiments (Table 14) also indicated that when the staphylococcal cells were grown in

Vitamin-free Casitone with added thiamine (2 μg/ml), niacin (0.05 μg/ml) and 6-aminonicotinamide (100 μg/ml), there was no inhibition of growth. Turbidity of 300 Klett units was reached within 17 hr of incubation (results not shown in the table). Analyses of the glucose catabolic pathways of cells grown under these conditions demonstrated that the oxidation of glucose via the HMP pathway was reduced to 0.3%, the lowest value ever recorded in the present study. Such inhibition in the HMP pathway activity by 6-AN has been previously shown in animal cells (22,40,41).

(2) Effect of 6-hydroxynicotinic acid on the TCA cycle activity of *S. aureus*. Analysis of various niacin analogs (Table 9) has indicated that some of the niacin analogs at high concentrations can completely replace niacin for stimulation of both growth and HMP pathway activity. None of the compounds affected the TCA cycle activity unless thiamine was present in the growth medium. The results of glucose catabolic pathways with the addition of 6-hydroxynicotinic acid (6HN) to the medium are shown in Table 15. The findings demonstrated that this 6-hydroxylated derivative of niacin, when added alone to the growth medium, did not stimulate staphylococcal growth. Similarly, the activities of the glycolytic pathways

were not increased. For instance, the growth within 17 hr reached a turbidity of 75 Klett units (not shown in Table 15) and the amount of glucose catabolized via the HMP pathway and the TCA cycle activity were 10.7% and 1.5%, respectively. When the staphylococcal cells were grown in Vitamin-free Casitone with added 6-hydroxynicotinic acid and thiamine, utilization of the HMP pathway remained less than 10% while the relative activity of the TCA cycle was increased by 3-fold (from 1.5 to 4.2%). It should be mentioned here that 6-HN or thiamine when added singly had no stimulatory effect on the TCA cycle; nor did the addition of 6-HN and niacin together to the growth medium increase the TCA cycle activity (Table 15).

Observation of staphylococcal cells previously cultivated in 2% Vitamin-free Casitone containing 1000 μg/ml of 6-HN (Table 15) and then incubated with niacin under resting conditions showed stimulation of both HMP pathway and TCA cycle activities. Under these conditions the amount of glucose oxidized via the HMP pathway was 20.5% and the relative activity of the TCA cycle was approximately 5.8%. As shown previously (Table 1) that addition of niacin alone did not stimulate the TCA cycle activity, but increases only in the HMP pathway were noted. This

TABLE 15

Alteration of the TCA cycle activity of *S. aureus* by 6-hydroxynicotinic acid (6-HN)

Addition to the 2% Vitamin-free Casitone growth medium	Addition to the resting cells grown in column 1 medium	TCA cycle activity (%)	% Glucose catabolized via EM	HMP (%)
6-HN (1000 μg/ml)	None	1.5	89.3	10.7
6-HN (1000 μg/ml)-niacin (2 μg/ml)	None	0.9	76.4	23.6
6-HN (1000 μg/ml)	Niacin (2 μg/ml)	5.8	79.5	20.5
6-HN (1000 μg/ml)-thiamine (2 μg/ml)	None	4.2	91.7	8.3
Niacin (2 μg/ml)	6-HN (1000 μg/ml)	0.7	78.5	21.5
Niacin (2 μg/ml)-thiamine (2 μg/ml)	None	4.5	81.6	18.4

Staphylococcal cells were grown for 17 hr in the medium shown in the 1st column, washed with and suspended in 0.05 M phosphate buffer. In some cases 6-HN or niacin were added, as indicated in the 2nd column, to the resting cells. The flasks were incubated for 2 hr and the estimation of glucose catabolic pathways was performed as described previously (see Methods).

indicated that 6-HN was acting like thiamine in stimulating the TCA cycle. The data from Table 15 also showed no increase in the TCA cycle if the cells were grown previously in Vitamin-free Casitone with added niacin and then incubated with 6-hydroxynicotinic acid.

(3) Effect of N-oxides of pyridine derivatives on growth and on glucose catabolism. Table 16 presents the results of the studies of various pyridine derivatives and their N-oxides on growth, HMP and on the TCA cycle activities of *S. aureus*. In general it was observed that all N-oxides tested were better growth stimulators than the corresponding parent compound. 3-Hydroxypyridine-N-oxide was an exception. For instance, addition of pyridine or 2-picoline did not stimulate either the growth or the metabolic activities of staphylococci. However, upon the addition of the corresponding N-oxides, pyridine-N-oxide and 2-picoline-N-oxide, growth was stimulated as was glucose utilization through the HMP pathway. The concentration of N-oxides required to yield maximal cell growth was generally lower than the concentration of the parent compounds or of other pyridine derivatives.

G. Uptake of ^{14}C-niacin and niacin analogs.

(1) Uptake of niacin-^{14}C by niacin-deficient *S. aureus*. Incubation of staphylococcal cells previously

TABLE 16

Effect of the addition of pyridine compounds and their N-oxides to a thiamine-supplemented Vitamin-free Casitone on staphylococcal growth and glucose catabolism

Compound tested (concn µg/ml)	Turbidity (17 hr) Klett units	TCA cycle activity (%)	% Glucose catabolized via	
			EM	HMP
Nicotinic acid (2 µg)	340	4.5	81.5	18.5
Nicotinic acid-N-oxide (100)	315	4.3	75.1	24.9
Nicotinamide (2)	342	4.2	80.9	19.9
Nicotinamide-N-oxide (100)	360	7.4	84.9	15.1
3-Pyridylcarbinol (100)	335	3.3	77.2	22.8
3-Pyridylcarbinol-N-oxide (500)	345	3.2	82.1	17.9
Pyridine (1000)	61	0.8	93.0	7.0
Pyridine-N-oxide (500)	330	2.2	84.3	17.7
2-Picoline (1000)	65	0.6	94.6	5.4
2-Picoline-N-oxide (1000)	340	3.0	80.2	19.8
3-Picoline-N-oxide (500)	360	4.4	81.5	18.5
3-Hydroxypyridine (1000)	55	1.1	93.7	6.3
3-Hydroxypyridine-N-oxide (1000)	96	1.5	89.5	10.5

% Glucose catabolized via the EM and the HMP pathways and the relative activity of the TCA cycle were estimated as described previously (see Methods).

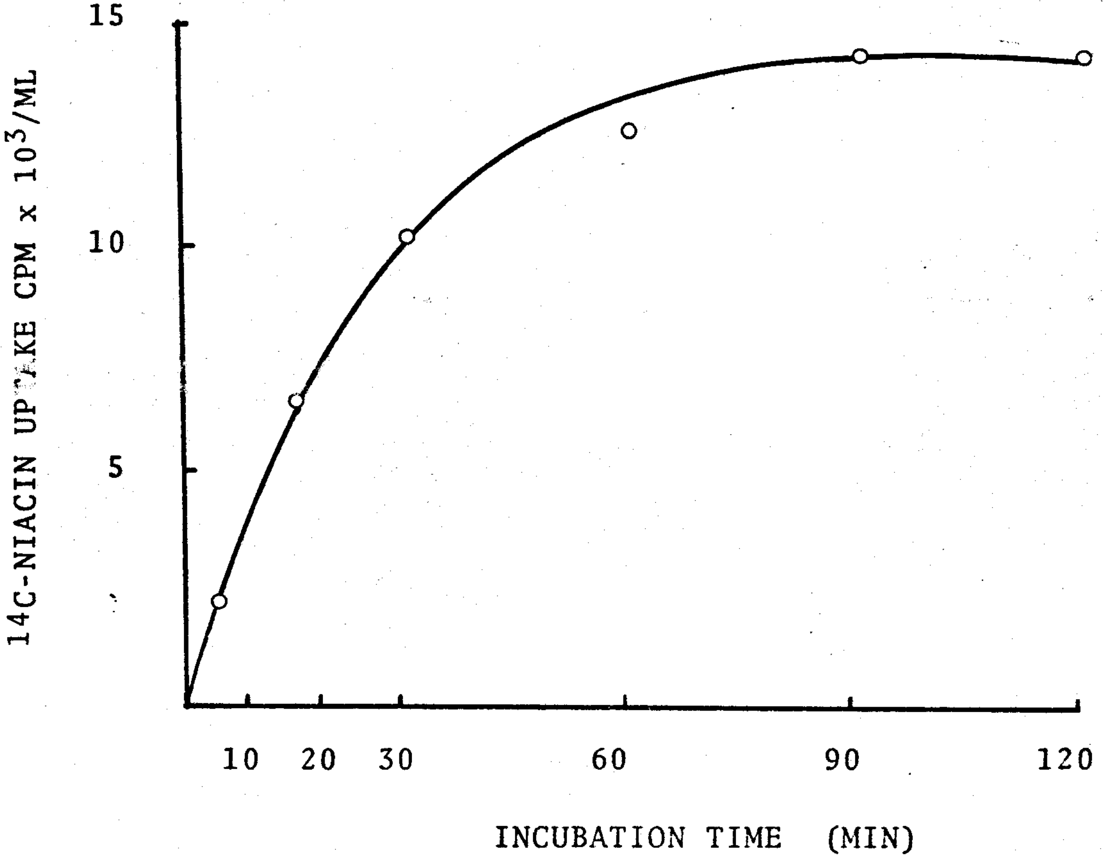

Figure 6. Uptake of ^{14}C-niacin by nonproliferating niacin-deficient S. aureus. Washed niacin-deficient cells equivalent to 3 mg (dry weight)/ml were incubated in buffered glucose containing ^{14}C-niacin, 5 μg/ml (114508 cpm/ml of niacin). The ^{14}C content of the cells was determined at the indicated time.

grown in unsupplemented Vitamin-free Casitone medium in reaction mixture at 37 C containing ^{14}C-niacin resulted in an accumulation of the radioactive vitamin within the cells (Fig. 6). Niacin uptake was found to be linear with time for about 30 to 40 min , reaching a maximum at 60 min. Further incubation, for periods up to 120 min , did not result in an additional uptake of the vitamin.

(2) pH Dependence of niacin transport. The results of studies on the uptake of niacin at different pH values are presented in Fig. 7. The vitamin uptake was strongly influenced by the pH of the medium, the optimum pH being 5.5. As the pH of the incubation medium was raised from 5.5 to 8.0, there was a reduction in the uptake of the vitamin. On the basis of these results it is concluded that niacin enters the staphylococcal cells by a pH sensitive mechanism, the optimum hydrogen concentration for niacin uptake being approximately 5.5.

(3) Temperature dependence of niacin transport. The results of studies on niacin uptake by staphylococci at different temperatures are shown in Fig. 8. The niacin uptake by resting cells of *S. aureus* was influenced by the temperature of reaction mixture. The maximum uptake was shown to occur at 50 C. Cellular adsorption of niacin was found to contribute little to the amount of niacin uptake, since brief

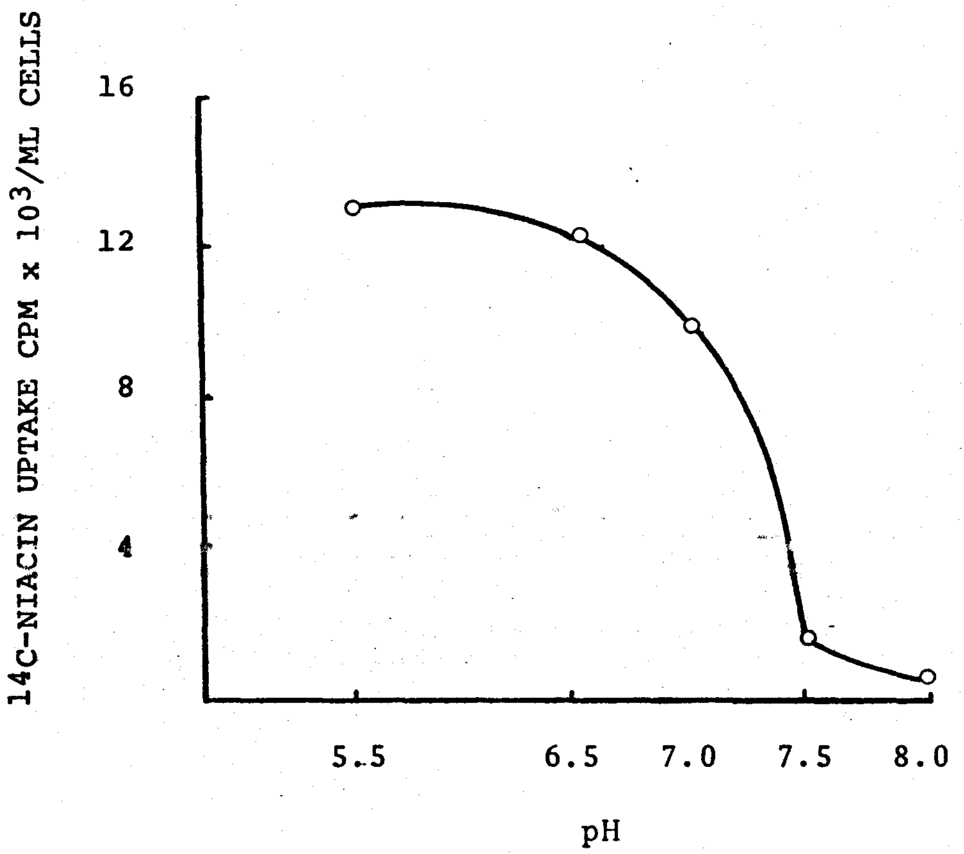

Figure 7. Effect of pH on ^{14}C-niacin transport in *S. aureus*. Conditions for the experiment were same as described in Fig.6 except that 0.2 M K phosphate buffer was used in the pH range from 5.5 to 8.0. The samples were removed at the end of 60 min for the estimation of radioactivity in the cells.

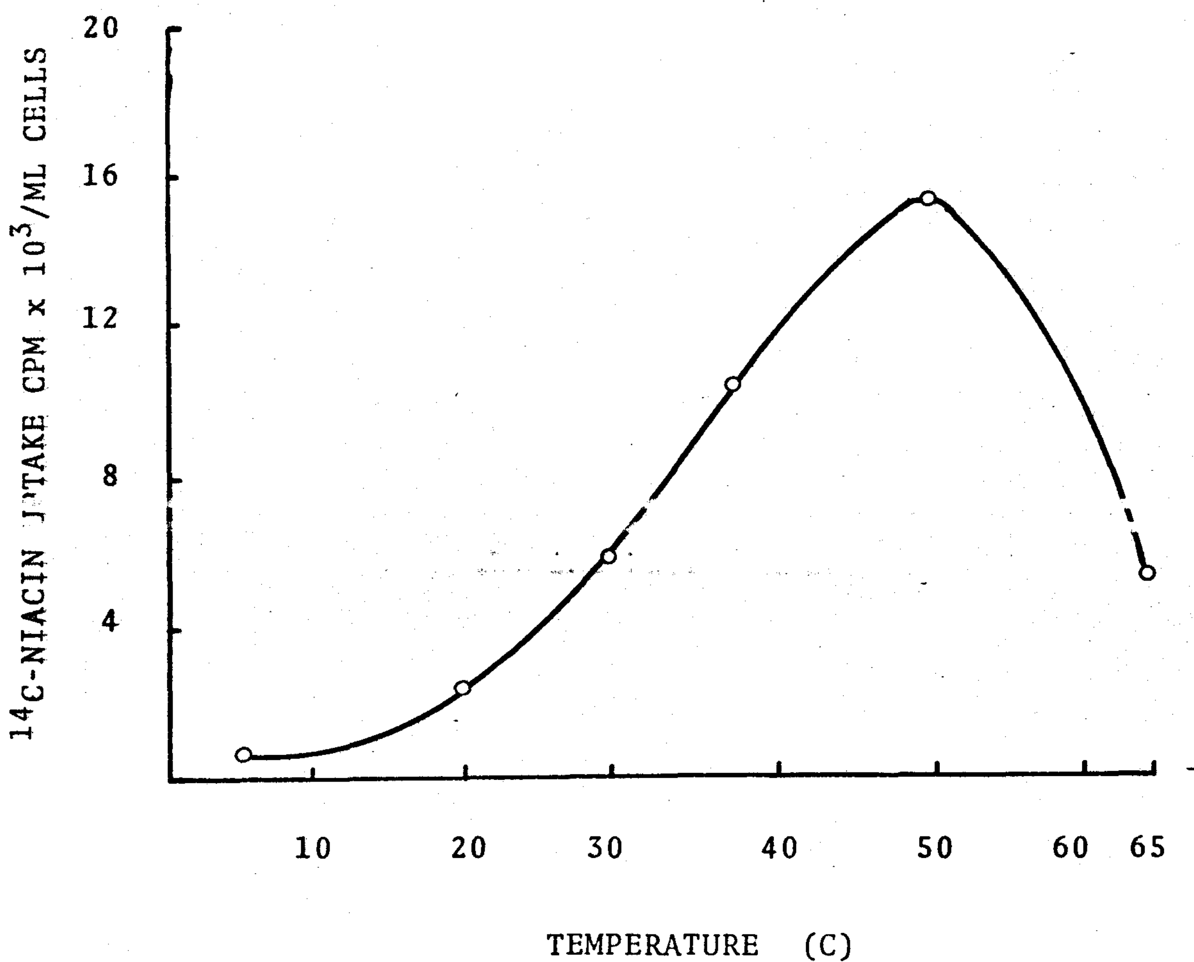

Figure 8. Effect of temperature on ^{14}C-niacin transport in s. aureus. The conditions for the experiment were same as described in Fig.6. The staphylococcal cells were incubated for 60 min at indicated temperature, and the radioactivity in the cells was determined as described previously (see Methods).

exposure of cells to niacin at 0-4C resulted in insignificant niacin uptake. Since energy generation within the cells is very low at this temperature, this also suggests that niacin uptake is energy dependent.

(4) Energy requirement. Many microbial transport systems are dependent upon, or are stimulated by, the presence of an energy source (43). In the experiments reported here, the addition of glucose to the medium slightly stimulated the uptake of niacin in vitamin-deficient staphylococcal cells (Fig. 9). Also, the addition of glucose to the incubation significantly stimulated niacin uptake in the staphylococcal cells previously grown in the presence of excess niacin (results are not included in the Fig. 9). The mechanism of stimulation of niacin uptake under this condition in the present study is not well understood.

(5) Effect of niacin concentrations. The results of experiments on the effect of niacin concentration on the transport system in staphylococci are presented in Fig. 10. The rate of niacin uptake increased with increasing niacin concentrations in the medium and tended to become saturated at 10^{-7} M.

(6) Inhibition of niacin uptake by niacin analogs. A measure of the specificity of the niacin transport system was obtained by investigating the effect of several

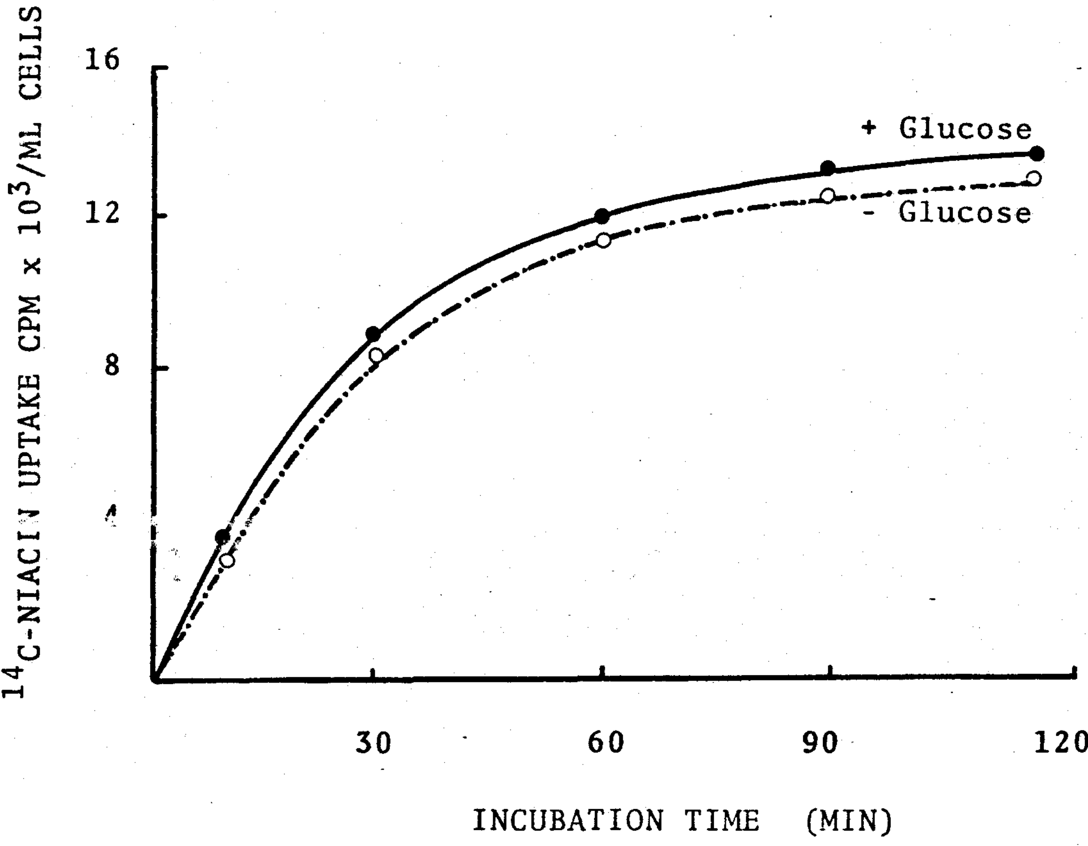

Figure 9. <u>Effect of glucose on ^{14}C-niacin transport in S. aureus</u>. Washed niacin-deficient cells equivalent to 3 mg (dry weight)/ml were incubated in K phosphate buffer (pH 7.0) containing ^{14}C-niacin, 5 μg/ml (114508 cpm/ml) and/or 1% glucose. Radioactivity in the cells was determined at various intervals as described previously (see Methods).

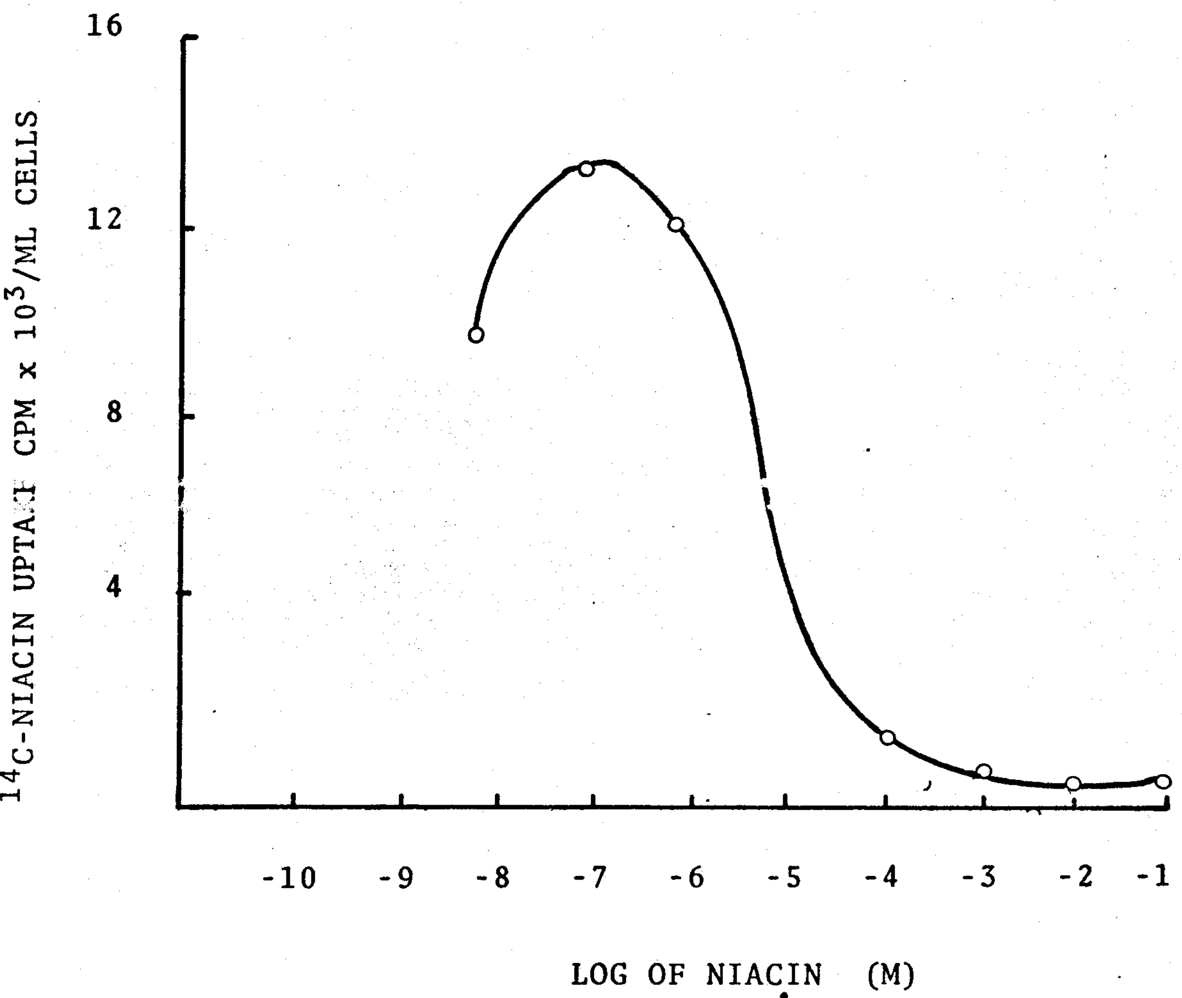

Figure 10. <u>Effect of niacin concentration on the uptake of niacin by niacin-deficient S. aureus</u>. Experimental conditions were as described in Fig. 6. Radioactivity in the cells was determined after 60 min of incubation.

structurally related compounds on ^{14}C-niacin uptake (Table 17). The presence of excess unlabeled niacin or of the pyridine analogs containing a substituant in the 3 position, 3-pyridylcarbinol, quinolinic acid, 3-hydroxypyridine, 4-chloronicotinic acid and 2-, 4-, and 5-hydroxynicotinic acid, inhibited the accumulation of radioactive niacin. However, pyridine-N-oxide and isoniazid, neither of which are substituted at the 3-position of the pyridine ring, did not inhibit the uptake process. Thus, the results indicated that niacin uptake by *S. aureus* is mediated by a transport system having structural specificity for niacin and closely related compounds.

(7) *Exchange between intracellular niacin and extracellular niacin and its analogs.* Many compounds which are accumulated by cells can be eluted from the intracellular pool by exchange with extracellular compounds of the same or similar structure (15). This has been cited as evidence for the existence of transport carrier systems in the cell membrane. In the present experiments (Table 18) the exchange between intracellular niacin and the extracellular niacin or niacin analogs did not occur. However, it should be pointed out that after niacin entered the cells, it was quickly converted to nicotinamide coenzymes NAD(P) and its intermediates and thus did not remain in a free state (46, 33).

TABLE 17

Inhibition of ^{14}C-niacin transport by niacin analogs

Unlabeled addition	Radioactivity in cell counts/min	% Inhibition ^{14}C-niacin uptake
None	12,850	0.0
Niacin	15	100.0
3-Pyridylcarbinol	110	99.1
Quinolinic acid	135	98.9
Pyridine-N-oxide	11,325	0.0
Isoniazid	11,765	8.5
3-Hydroxypyridine	55	99.6
2-Hydroxyniacin	75	100.0
4-Hydroxyniacin	74	100.0
5-Hydroxyniacin	60	99.5
4-Chloronicotinic acid	905	91.0

Staphylococcal suspensions containing 3 mg (dry weight) of cells in 0.05 M K phosphate buffer, pH 7.0 and 1% glucose were exposed simultaneously to 2 µg/ml of ^{14}C-niacin (114508 cpm) and 2 mg of unlabeled analog. After 30 min, the cell suspensions were filtered, washed and then counted, as described previously (see Methods).

TABLE 18

Exchange between intracellular ^{14}C of cells preloaded with ^{14}C-niacin and with unlabeled extracellular niacin or niacin analogs

Radioactivity (cpm/ml, cells) after addition of unlabeled					
Time min	Buffer	Niacin	Quinolinic acid	3-Pyridyl-carbinol	Isoniazid
0	12,110	12,545	11,500	10,907	12,041
30	12,049	11,961	11,509	11,595	11,157
60	11,570	11,106	11,916	10,751	10,569
120	11,618	10,671	10,253	10,807	11,326

Cells preloaded with ^{14}C-niacin as described previously (see Methods), were added at a concentration of 3 mg/ml (dry weight) to flasks containing 0.05 M potassium phosphate buffer pH 7.0, 1% glucose and either unlabeled niacin or niacin analogs at a final concentration of 2 mg/ml. Cells were incubated at 37 C, samples removed at intervals and the cellular radioactivity was determined.

(8) Uptake of labeled quinolinic acid and isoniazid by niacin-deficient S. aureus. Staphylococcal cells took up comparatively little isoniazid or quinolinic acid. Fig. 11 and 12 show the uptake of two different concentrations of isoniazid and quinolinic acid by staphylococcal cells under resting cell conditions. As the external concentration of radioactive quinolinic acid was raised to 500 μg/ml, the rate of uptake was found to be slightly increased during the 2 hr incubation period. The uptake, although low (1.0%), was found to be maximal at a concentration of 500 μg/ml (Fig. 12). Similarly, small differences were observed in the uptake of isoniazid by increasing the external isoniazid concentration from 10 to 500 μg/ml in the medium (Fig. 11). The uptake of isoniazid was also time-dependent, as more ^{14}C-isoniazid entered the cells at the end of a 6 hr compared to a 2 hr period. Analyses of the glucose catabolic pathways under identical conditions showed that there was an increase in the utilization of the HMP pathway from 6.2 to 20.4% when the concentration of isoniazid was increased from 10 to 500 μg/ml. Similar results were obtained with quinolinic acid (results not shown in Fig. 11 and 12). Therefore, these results indicated that although the uptake of the niacin analogs isoniazid and quinolinic acid was low (2%), it was sufficient to bring about the metabolic changes similar to those caused by niacin.

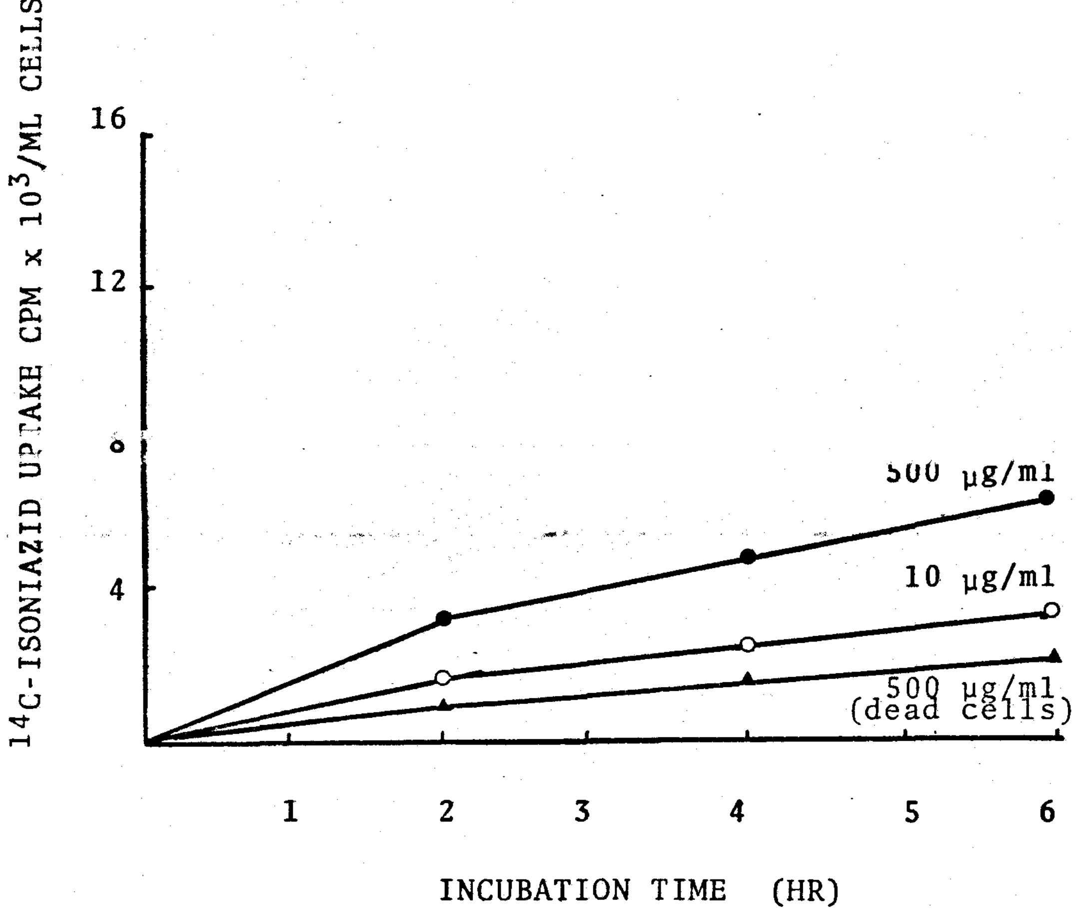

Figure 11. <u>Uptake of isoniazid-^{14}C by nonproliferating niacin-deficient S. aureus</u>. Cells equivalent to 3 mg (dry weight)/ml were incubated at indicated time, in reaction mixture containing either 10 or 500 μg/ml of isoniazid-^{14}C (222635 cpm/ml, final concentration). The uptake of the analog by the cells was determined as described previously.

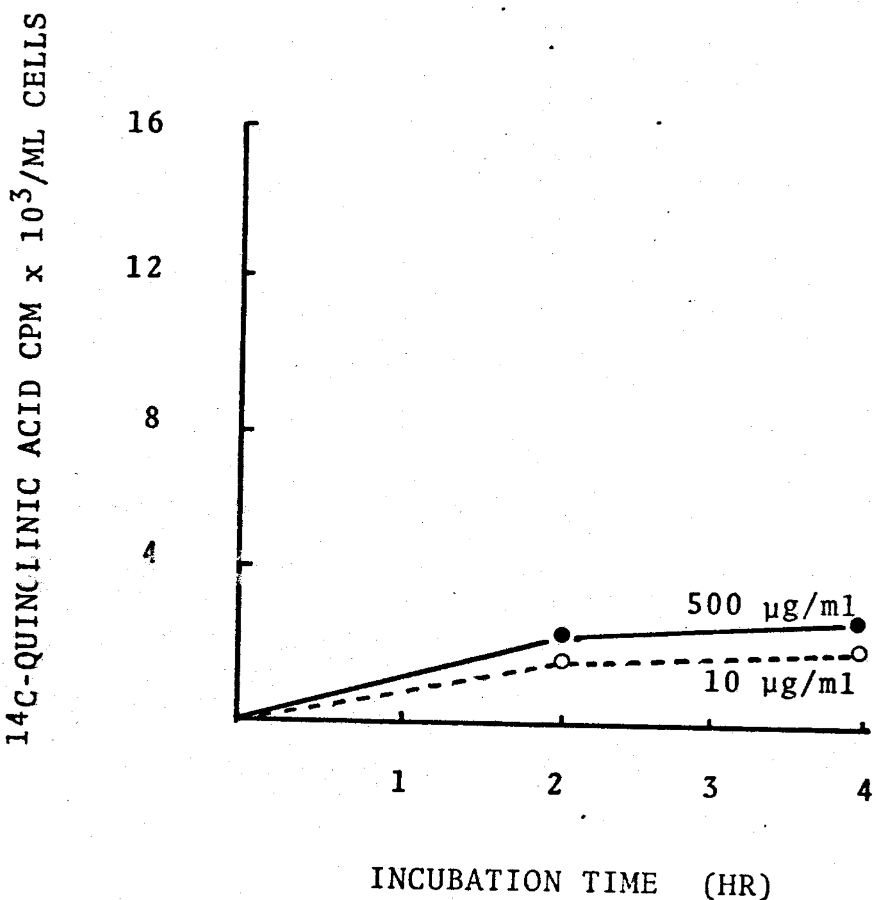

Figure 12. Uptake of quinolinic acid-^{14}C by nonproliferating niacin-deficient S. aureus. Experimental conditions were as described in Fig.11 except isoniazid was replaced with quinolinic acid-^{14}C.

IV. DISCUSSION

In the present study an attempt has been made to investigate some of the factors that might control the degree to which the glucose catabolic pathways of staphylococci are modified, as a result of the addition of either niacin and /or thiamine to the growth medium.

The importance of an adequate supply of niacin and thiamine in glycolysis (i.e., the conversion of glucose to pyruvate or lactate without regard to metabolic pathways) has been studied in staphylococci by Kligler et al. (61). According to these investigators 2.5 times more glucose was used when both vitamins were present in the growth medium than was used when niacin alone was present. Results from the present study have also indicated that there was an increase in the rate of glycolysis when these vitamins were added to certain vitamin-deficient growth media (Fig. 2), although addition of thiamine alone to the medium did not result in stimulation of glucose utilization. Thus, in the present study there were no differences in glucose utilization by staphylococci grown in a medium containing either niacin or niacin and thiamine. The difference between these results and those of Kligler et al (61) perhaps stems from the use of resting cells in the present studies, previously grown in the presence of vitamins, whereas Kligler et al. (61) analyzed glucose utilization from the medium by actively growing cells.

In the present study it was found that, following the addition of niacin or niacin and thiamine to Vitamin-free Casitone, 18 to 22% of the glucose was oxidized via the HMP pathway by the cells. Surprisingly, the addition of thiamine alone to the growth medium did not result in a stimulation of the glucose oxidation through the HMP pathway (Table 1). This was an important finding since thiamine, in the form of diphosphothiamine (DPT), is required as a coenzyme in this pathway. If the DPT coenzyme is missing or is present in amounts that limit the expression of the full activity of the enzyme, the effect would be the same as the absence of the enzyme, with a resultant block in the activity of the HMP pathway. It has been shown that the addition of thiamine to thiamine-deficient red blood cells stimulated transketolase activity and HMP pathway, indicating that transketolase was the rate-limiting step in the HMP pathway of red blood cells (102). A similar limiting role for transketolase in both yeast and rat liver has also been demonstrated (6, 87), and the addition of thiamine has induced transketolase in yeast (124).

Data from this dissertation have shown that when thiamine was added to the medium, there was no stimulation of glucose oxidation through the HMP pathway in staphylococci. The lack of the HMP stimulation by added thiamine may perhaps reflect that there was already present an excess of the vitamin for transketolase activity. On the basis of these findings alone, it is

not possible to exclude the rate-limiting or regulatory role of DPT-linked transketolase in the HMP pathway of S. aureus. However, glucose oxidation via the HMP pathway increased markedly following addition of niacin, without any thiamine and under the conditions that precluded enzyme formation (Table 5). It may be concluded that the activity of DPT-linked transketolase is not limiting the activity of the HMP pathway under these conditions.

All living cells contain thiamine, if it is not supplied exogenously then the cell must synthesize it. Although, thiamine was originally considered to be a vitamin required for the growth of staphylococci (62), Idriss and Blumenthal (48) obtained moderate growth of staphylococci in a niacin-supplemented synthetic medium, free of thiamine. The low rate of growth and increased HMP activity were consistent even after repeated transfer of the cells in the absence of thiamine. Based on these findings, they concluded that there must be a limited endogenous synthesis of thiamine in the cells. Since the results of radiorespirometric studies with the cells grown in niacin supplemented growth medium lacking thiamine have shown that the stimulation of the HMP cycle can occur in the absence of added thiamine, then it is clear that the staphylococci can synthesize enough thiamine to allow the HMP pathway to act at a maximal rate. Thus, thiamine is not limiting the activity of the HMP pathway in staphylococci as it does in the red blood cells of animals.

When staphylococci were grown in a vitamin-deficient medium (2% Vitamin-free Casitone), with either added niacin or thiamine alone, there was no stimulation of the TCA cycle activity. Increased TCA cycle activity (10-fold) was only detected when both niacin and thiamine were added to the growth medium. The mechanism of this stimulation, only after the addition of both niacin and thiamine, is not presently understood, although the coenzymes forms of both vitamins are required for the activity of a number of enzymes in the TCA cycle.

Relatively large variations in the degree of utilization of the glycolytic pathways of staphylococci were observed when the cells were grown in different growth media. For example, when staphylococci were grown in Vitamin-free Casitone containing both vitamins, the amount of glucose catabolized via the HMP pathway was 18% as compared to a value of 35% when the cells were grown in synthetic medium supplemented with niacin and thiamine (Table 1). Similarly, it had been shown that the addition of niacin and thiamine to Trypticase growth medium (which contains glucose) yielded cells that oxidized 46% of the glucose via the HMP pathway (81). Thus, these results demonstrate that the relative degree of utilization of the HMP pathway in *S. aureus* is significantly affected by the nature of the growth medium. At present, the reasons for the absolute differences in the degree of utilization of the glycolytic pathways of staphylococci grown in different growth media are not fully understood. However, it

is clear in all instances studied that the addition of niacin or niacin and thiamine to the growth medium (Vitamin-free Casitone, synthetic medium or Trypticase broth) results in the growth of cells which have the property of oxidizing increased percentages of glucose via the HMP pathway. To minimize complications resulting from the use of complex media, Vitamin-free Casitone medium was used for most of the experimental work.

Until now we have considered the percentage of glucose molecules metabolized by the EM and by the HMP pathways. It is possible that one may receive a distorted view if the actual amounts of glucose traversing these pathways are not considered. For example, in mutants of *Salmonella typhimurium* and of *Escherichia coli*, deficient in phosphoglucose isomerase, the EM pathway was found not to operate efficiently because of this enzyme deficiency and the rate of both growth and glucose utilization was only about 20% of that noted in the parent strain. Although, the mutants used less total glucose, the rate of utilization of glucose via the HMP pathway (3 μmoles/mg/hr) was approximately the same as that observed in the parent strains (29, 30). Consequently, the percentage of glucose oxidized by the HMP pathway increased, but only because the amount going through the EM pathway decreased. An analysis of the results with staphylococci indicated that an analogous situation occurred when the percentage of glucose being oxidized via the HMP pathway

increased after the addition of niacin. The difference was that the amount of glucose being oxidized via the EM pathway remained relatively constant (or increased slightly) while the amount oxidized via the HMP pathway increased significantly.

The usual percentage of glucose catabolized via the HMP pathway in niacin-deficient cells is 6%, and since there are only 2 pathways, by difference, the amount catabolized via the EM pathway is 94%. About 33% more glucose was used by niacin-rich cells than was used by cells grown in the absence of the added vitamins, and one might ordinarily expect this extra glucose to be catabolized by the two glycolytic pathways in the same relative proportions. However, the results of the isotopic experiments (Table 1) indicated that in niacin-rich staphylococci, the amount of glucose oxidized via the HMP pathway increased to about 20% while, by difference, the amount oxidized via the EM was reduced to 80%.

In niacin-rich cells the actual amounts of glucose traversing the EM pathway each hour was 9.6 μmoles/hr (12 μmoles glucose/hr x 80% glucose oxidized via EM = 9.6) and by difference, since a total of 12 μmoles/hr were used, the amount of glucose oxidized via the HMP must be 2.4 μmoles/hr. In a similar manner, vitamin-deficient cells used 8.5 μmoles/hr via the EM pathway (9 μmoles/hr x 94% glucose oxidized via EM = 8.5) and by difference, since a total of 9 μmoles of glucose/hr were used, the amount of glucose

oxidized via the HMP pathway was 0.5 μmole/hr. Thus the inclusion of niacin in the growth medium resulted in a relatively slight increase in the amount of glucose utilized via the EM pathway (from 8.5 to 9.6 μmoles/hr), while there was a 5-fold increase (from 0.5 to 2.4 μmoles/hr) in the glucose oxidation through the HMP pathway. Although the addition of niacin resulted in a decreased percentage of glucose being metabolized via the EM pathway (from 94% to 80%), the actual amount of glucose used via this pathway increased slightly. The increased rate of glycolysis in niacin-rich cells was primarily due to the additional passage of about 2 μmoles of glucose through the HMP pathway. Thus, the total amount of glucose utilized via the EM pathway remained essentially the same in both niacin-rich and niacin-deficient staphylococcal cells. This is the reverse of the findings by Fraenkel (29) in enteric bacteria where the bacterial mutants, with deficiencies of an EM enzyme, had significantly reduced amounts of glucose being oxidized via the EM pathway but relatively constant amounts via the HMP pathway.

When one disintegrates staphylococci and assays the levels of the individual glycolytic enzymes _in vitro_ under conditions that are optimal for substrate and coenzyme concentrations, such assays do not necessarily yield information about the activity of these enzymes within the cells. These analyses, however, reveal the potentialities (i.e., the maximum activity of that

enzyme under optimal conditions) of the individual enzymes. Enzymes which play a regulatory role in determining the proportion of glucose that is metabolized via one of the two pathways will also suffer the same limitations during in vitro assays. Consequently, an attempt was made throughout the present study to use both in vivo and in vitro techniques to estimate the activity of a metabolic pathway.

The pattern of $^{14}CO_2$ production from ^{14}C-labelled glucose by staphylococci which had been grown in media supplemented with both vitamins, strongly indicated the stimulation of both the HMP pathway and the TCA cycle. Measurements of the enzyme activities suggested that the increased activities of the glycolytic pathways were due to the synthesis of increased amounts of enzymes. Thus, there were striking and consistent increases in the activity of G6PD and 6PGD when the HMP pathway operated maximally and similar increases in ICD were noted when the TCA cycle activity was stimulated (Table 1 and 2). Under various growth conditions, the activities of the two HMP enzymes (G6PD and 6PGD) were shown to be closely related to the level of staphylococcal glucose catabolism via the HMP pathway, as indicated by the ability to convert C_1 of glucose to CO_2. A similar relationship was found between ICD levels and the TCA cycle activity, as indicated by the release of CO_2 from C_6 of glucose.

The markedly increased levels of G6PD (ca. from 45 to 160 units) that were found in staphylococci following the additon of the vitamins to the growth medium were accompanied by a tripling in the percentage of glucose oxidized via the HMP pathway in S. aureus (Table 2 and 3). Such increased utilization of the HMP pathway and accompanying increased content of HMP enzymes have also been observed in yeast and mammalian systems (80, 87, 109).

Experiments with cell-free extracts, in which the individual enzyme may be added to those already present in the extract, have also yielded some useful information about the role of individual enzymes in controlling the activity of a pathway. For example, the addition of phosphohexose isomerase and glucose-6-phosphate dehydrogenase (G6PD) to a hemolysate of normal red blood cells resulted in an increase in the amounts of glucose metabolized via both the EM and the HMP pathways. When hemolysates were prepared from G6PD deficient blood cells, added phosphohexose isomerase was without any apparent effect, indicating that the enzyme was already present in excess, whereas the addition of G6PD caused a 4-fold increase (from 13.1 to 45.8%) in the amount of glucose oxidized by the HMP pathway, thus confirming its rate-limiting role. It should be noted that these experiments with cell-free extracts were carried out on the presence of optimal concentrations of NADP required for the HMP

pathway stimulation. Since the present studies with staphylococci resulted in the conclusion that the enzymes were not limiting the rate of glucose oxidation via the HMP pathway, similar experiments were not performed.

The results of the present experiments indicated that when staphylococci were grown in supplemented Vitamin-free Casitone, increased G6PD activity was always paralleled by increased HMP pathway activity (Table 2 and 3). From this observation it appeared that the level of G6PD in some manner limited the activity of the HMP pathway in staphylococci.

The first indication that the enzyme levels were probably not the actual limiting factors for the operation of the HMP pathway in intact staphylococcal cells, came from the results of experiments with cells grown in synthetic medium. About 35% of the glucose was metabolized via the HMP pathway when *S. aureus* was grown in synthetic medium containing excess amounts of niacin and thiamine, compared to only 18% when these vitamins were added to Vitamin-free Casitone (Table 1). In spite of the increased level of activity of the HMP pathway in the intact cells, the activities of G6PD and 6PGD, two of the key enzymes in the HMP pathway, from extracts derived from the synthetic medium, were only 2/3 as much as those derived from cells grown in Vitamin-free Casitone with added niacin and thiamine (Table 2). Therefore, it appeared that increased amounts of the glycolytic

enzymes were not necessary for more active operation of the HMP pathway in staphylococci. A similar situation existed for the TCA cycle.

In order to get more direct information on the role of enzymes in the regulation of the HMP pathway, we undertook experiments with resting cells. Under resting cell conditions protein synthesis in the cells is minimized due to the absence of a nitrogen source in the incubation medium. Although some turnover of protein is expected in the cells, there can be no net increase in protein synthesis. Thus under resting cell conditions the effect of vitamins on metabolic pathways can be examined with minimal changes in enzyme levels.

Radiorespirometric studies showed that the incubation of vitamin-deficient staphylococci, possessing a relatively low percentage of glucose oxidation via the HMP pathway, with the vitamins in the buffered-glucose medium converted them to staphylococci with glycolytic characteristics similar to those of cells that had been grown in the presence of the vitamins (Table 4). The amount of glucose catabolized via the HMP pathway was approximately 19%, whether the vitamin supplementation had been made under growing or resting cell conditions. The stimulatory effects of the vitamins on the activity of the TCA cycle were similarly observed. Enzyme analyses indicated that the levels of the two key HMP enzymes were only 30% of

those observed under growing conditions, even in spite of a greater utilization of glucose via the HMP pathway (Table 3 and 5). This clearly indicated that the HMP enzymes G6PD and 6PGD were not limiting the activity of the HMP pathway *in vivo*.

To further ensure that protein synthesis was not proceeding in the resting cells as a result of the utilization of amino acids from either an endogenous amino acid pool, and/or from a low level of protein turnover, one of the following three antibiotics, inhibitors of protein synthesis, chloramphenicol, actinomycin D or puromycin, were added along with the vitamins at concentrations ranging from 6 to 100 μg/ml. Growth of the majority of strains of *S. aureus* is completely inhibited by chloramphenicol and actinomycin D at concentrations of 6 and 0.6 μg/ml, respectively (120). The findings of the experiments were essentially the same as those with the resting cells suspensions, namely, that none of the antibiotics were able to prevent the stimulation of the HMP pathway or the rise in the levels of NAD and NADP, following the addition of niacin to the vitamin-deficient staphylococci. Furthermore, the enzyme levels in the HMP pathway did not increase in the cells with the increased HMP activity (Table 6). Surprisingly, it was found that actinomycin D actually stimulated utilization of the HMP pathway and also increased NAD level. The mechanism of this stimulation by actinomycin D is not presently understood and was not investigated further.

Based on the results just discussed it was concluded that the stimulation of the HMP pathway caused by the addition of niacin was not related in any way with the synthesis of enzymes.

Since the levels of the glycolytic enzymes within the cells are not the rate-limiting factors in staphylococcal carbohydrate metabolism and do not play a regulatory role in the HMP pathway of S. aureus, any changes in enzyme levels within cells resulting from the addition of the vitamins to the growth medium are probably secondary to the changes in nicotinamide coenzyme levels. This will be discussed further in this section.

In contrast to the present results with staphylococci, the levels of enzymes have been claimed to play a significant role in determining the degree to which the EM and HMP glycolytic pathways are utilized in yeast (87). The findings with the yeast were based on the fact that when yeast were grown with nitrate as the nitrogen source, instead of with amino acids as the nitrogen source, there was an increase in the levels of the enzymes, G6PD and transketolase, along with increased $^{14}CO_2$ production from glucose-1-^{14}C, indicating more active operation of the HMP pathway. It was also noted that the addition of the antibiotic, cycloheximide, to the growth medium prevented both new enzyme synthesis and increased activity of the HMP pathway. From these findings, it was concluded that new enzyme synthesis

was necessary for increased operation of the HMP pathway and that there was a strong correlation between the level of G6PD and the degree of operation of the HMP pathway in yeast.

In the present study, it was also found that the levels of G6PD and 6PGD paralleled the degree of utilization of the HMP pathway in staphylococci under growing conditions (Table 3). However, in the present study suitable experimental conditions were found which demonstrated that the increased utilization of the glucose through the HMP pathway, as a result of the addition of vitamins, occurred without the synthesis of these enzymes (Table 3). On the basis of these findings, it is concluded that new enzyme synthesis is not necessary for maximum operation of the HMP pathway in staphylococci. This may just represent examples of different controlling factors in different microbes.

The measurement of enzyme levels in the cells that had been incubated with chloromphenicol eliminated the possibility of any enzyme formation in the present study through activation or assembly of polypeptide precursors, such as is thought to occur in the formation of enterotoxin B by S. aureus, even in the presence of chloramphenicol (74).

Induction of G6PD synthesis by coenzymes, vitamins and metabolites has been observed in animal and microbial cells (3, 105, 123, 124). For instance, thiamine in the form of diphosphothiamine induced the synthesis of pyruvate decarboxylase

in yeast, with resultant increase in glycolysis (123). This finding was based on the observation that pyruvate decarboxylase activity increased by 50% during the incubation of yeast cells with glucose and thiamine and by 300% when the cells were incubated with glucose, thiamine and a nitrogen source. Similarly, an increase in the pyruvate decarboxylase activity was prevented by the inclusion of proflavine, p-fluorophenylalanine or 5-fluorouracil in the incubation medium. These results indicated that the higher activity of pyruvate decarboxylase after incubation with thiamine was due to the stimulation of *de novo* synthesis of the enzyme and not an activation of pre-existing enzyme molecules. Later studies demonstrated that thiamine also induced transketolase (124). There are also other examples which support the finding that cofactors of an enzyme, in addition to regulating the activity of the existing enzyme, may influence the amount of the protein moiety of the same enzyme that is found *in vivo* (37). However, in *S. aureus*, there was no increase in the levels of the glycolytic enzymes, when the vitamins were added under resting cell conditions, although the glycolytic pathways were maximally stimulated (Table 4). The additional use of antibiotics in the experiments (Table 6) eliminated any possibility of new protein synthesis occurring in the cells. Based on these results it can be concluded that there was no induction of glycolytic enzymes in *S. aureus* by the added vitamins.

Since the enzyme levels were not responsible for regulating the relative amounts of glucose being metabolized by the EM and the HMP pathways in staphylococci, we next considered the possible regulatory role of NAD and NADP levels. NADP has been shown to be an important regulator of the HMP cycle in various microbial and mammalian systems (24). In numerous *in vitro* experiments it has been shown that the addition of either electron acceptors which would reoxidize NADPH to NADP or of enzyme systems capable of converting NADPH to NADP, resulted in increased glucose oxidation via the HMP pathway (121). Thus the NADP concentration and/or the rate of NADPH reoxidation to NADP are usually limiting the overall rate of glucose degradation via the HMP pathway.

In cooperative studies with R. Hoo (46) in this laboratory, it was observed that the levels of nicotinamide coenzymes in staphylococci always increased in parallel with increased levels of glucose oxidation via the HMP pathway following the addition of niacin either under growing or resting cell conditions (Table 6). For instance, it was demonstrated in staphylococcal cells that when niacin was added to the growth medium, there was a 15-fold increase in NAD and a 2-fold increase in NADP levels at the same time there was a 3- to 4-fold increase in glucose oxidation via the HMP pathway. This suggested that either the levels of NAD and/or NADP played a regulatory role for the amount of glucose oxidation via the HMP pathway.

The regulatory role of NADP in the activity of the HMP pathway in various microorganisms has been well established (14). However, NAD has not been previously proposed to be involved in controlling the overall activity of the HMP pathway. It is difficult to imagine how NAD would control some of the enzymatic reactions of the HMP pathway since NAD does not serve as a coenzyme in this pathway, with the exception of few microbes (13, 125). In staphylococci, the enzymes G6PD, 6PGD and ICD all had a specific coenzyme requirement for NADP in the three assay systems. It should be noted that only crude cell-free extracts served as enzyme sources in the present studies. The addition of NAD to the assay mixture which contained an excess of NADP (0.1 mM) did not influence the rate of enzymatic reactions. These results suggested that the NAD levels did not stimulate the activity of HMP enzymes *in vitro*, and therefore, it appears that the role of NAD in regulating the HMP pathway in staphylococci, is probably due to some mechanism other than the direct stimulation of G6PD and 6PGD activities.

Nozawa and Mizuno (86) observed a reduction in the intracellular NAD level in niacin-requiring *E. coli* during the course of "starvation". The NAD content decreased to one tenth of normal level after two hr of incubation of washed cells in buffered glucose. Therefore, similar experiments were designed to determine whether such a variation in NAD could be observed

in staphylococci during starvation and, if so, to correlate the NAD and NADP levels with the degree of utilization of the two glycolytic pathways.

Incubation of washed resting staphylococcal cell suspensions that had previously been cultivated in Vitamin-free Casitone containing niacin (0.5 μg/ml), resulted in a reduction in the HMP pathway from 18% to 10%, while the intracellular NAD level decreased from 2.2 to 1.5 μmoles/g (Fig. 3). During this incubation for 6 hr in the vitamin-free buffered glucose solution, while the NAD level decreased, the NADP level remained constant or increased very slightly. However, the addition of niacin (100 μg/ml) to the incubation medium at the end of the 6 hr period led to a simultaneous increase in the HMP pathway utilization (to 19%) and the NAD content (to 4 μmoles/g) without a corresponding increase in the NADP level (Fig. 3). Replacing niacin with pyocyanin (an electron acceptor) in the incubation medium also resulted in an increase in the utilization of the HMP pathway (from 10 to 22%) without increasing the coenzyme levels. These results suggested that NAD and pyocyanin probably acted in the same manner while stimulating the activity of the HMP pathway, by reoxidizing NADPH to NADP. In the case of pyocyanin, the action was directly on the NADPH, while the NAD probably acted via the transhydrogenase system with NAD being reduced to NADH during the concomitant oxidation of NADPH to NADP.

On the basis of these results it appeared that a certain intracellular NAD level was necessary, in addition to the level of NADP, in order to allow this expanded operation of the HMP pathway in S. aureus. Under the conditions studied here, with a relatively constant NADP level of about 0.4 µmoles/g, as the NAD concentration was reduced to a value below 2.2 µmoles/g, the HMP pathway ceased to operate maximally. The importance of the NAD level in allowing the augmentation of glucose oxidation via the HMP pathway was further substantiated by the finding that when the NAD level was restored to 2 µmoles/g following the addition of niacin at the end of the 6 hr incubation period (Fig. 3), the HMP pathway once again returned to its maximal level. These results suggested that the NAD level in some manner played a regulatory role in the degree of operation of the HMP pathway in staphylococci.

In highly oxidative organisms such as Pseudomanas saccharophila and Pseudomonas fluorescens, which predominantly utilize the HMP and/or ED pathways, with little or no EM pathway, intracellular levels of NADP are relatively low. For example, the level of NADP in these two organisms 0.8 µmoles/g and 0.04 µmoles/g, respectively (71). In such organims the potential rate-limiting effect of NADP on the activity of the HMP and ED pathways has been overcome by the presence of active NADPH oxidase and of transhydrogenase (24) resulting in a very rapid reoxidation of NADPH to NADP.

In S. aureus the level of NADP was found to be in the range of 0.6 μmoles/g when the HMP pathway was being utilized maximally (45). This level of NADP was similar to those found in other facultatively aerobic and anaerobic microbes, such as E. coli, B. subtilis and P. saccharophila (71). In the present study with S. aureus no definite correlation was found between the transhydrogenase and NADPH oxidase activities and the degree of utilization of the HMP pathway. The low activities of these enzymes (Table 5) in S. aureus could be explained on the basis that the EM pathway still served as a major glucose catabolic route (78%), even when the HMP pathway operated maximally (22%). Therefore, the low levels of transhydrogenase and of NADPH oxidase, found in S. aureus, were probably sufficient to generate NADP for the HMP pathway activity. Such is the case in B. subtilis and also in E. coli, which do not use the HMP pathway as the major route for glucose catabolism and which also contain relatively low amounts of NADPH oxidase and transhydrogenase (24). Furthermore the finding that the expanded activity of the HMP pathway in S.aureus required a NAD level of greater than ca. 2 μmoles/g can best be interpreted to mean that the transhydrogenase system is involved in NADP regeneration even in the facultative anaerobes such as S. aureus. To our knowledge this is the first instance that the experimental evidence supports this concept in any microbe other than the strict aerobes such as pseudomonas.

One of the major conclusions drawn from the experiments just discussed was that the addition of niacin to the growth medium stimulated the glucose utilization via the HMP pathway by increasing the cellular levels of both the glycolytic enzymes and the nicotinamide coenzymes, NAD(P). However, through the use of resting cell conditions, it was demonstrated that the increased levels of enzymes were secondary effects and that certain intracellular NAD and/or NADP levels were required for the regulation of the HMP pathway in S. aureus.

The results of the present experiments have indicated thus far that either the level of NAD and/or NADP may be involved in the control mechanism regulating the degree to which glucose is oxidized via the HMP pathway in staphylococci. In order to obtain staphylococcal cells in which there were further variations in the coenzyme levels, some niacin analogs were employed in the present studies. The niacin analogs isoniazid and 6-aminonicotinamide have been known to reduce the intracellular levels of nicotinamide coenzymes in both microbial and mammalian systems (40, 41, 122). Surprisingly, it was found in the present studies that many of the niacin analogs were able to completely replace the niacin requirement for staphylococcal growth. Therefore, further studies with the analogs were pursued.

Previous investigations with niacin analogs had indicated that quinolinic acid, picolinic acid, isonicotinic acid, nicotinyldiethylamide, and certain other pyridine derivatives, when added to the growth medium at concentrations of 10^{-4}M (ca. 0.15 μg/ml), could not replace niacin as a growth factor for staphylococci (62, 68, 69). These failures to replace niacin and to permit growth were not due to the toxicity of the compounds since staphylococcal growth resumed following the addition of nicotinamide to media containing the various compounds. The results of the present studies, which are summarized in Table 10, confirmed these findings when the analogs were used at concentrations of 10-20 μg/ml. However, when the concentrations of the analogs in the growth medium (containing thiamine) was increased from a level of 10 to a level of 100 or 1000 μg/ml all of the analogs in Group I (Table 10) stimulated staphylococcal growth to approximately the same degree as niacin. For example, 3-AP or PSA added to a thiamine-supplemented 2% Vitamin-free Casitone growth medium at a concentration of 10 μg/ml produced a turbidity of 70 Klett units, approximately the same as that of the control. However, when the concentration of the analogs was increased to 1000 μg/ml in the medium, the turbidity increased to 343 Klett units. This compares to a value of 340 Klett units when niacin was used at a concentration of 2 μg/ml. Similar results were obtained with most of the other analogs from

Group I (Table 10). In the absence of added thiamine, however, the growth stimulation was either absent or minimal (Fig. 4 and Table 7), even when high concentrations of niacin analogs were used.

When analogs from Group II (Table 10) were added to a thiamine supplemented Vitamin-free Casitone medium even at concentrations of 1000 μg/ml, they were unable to stimulate growth maximally. For example, when either 6-chloronicotinamide or picolinic acid was added to the growth medium at a concentration of 1000 μg/ml turbidity readings of only 170-180 Klett units were obtained. This amount of growth was only approximately 2/3 as much as the amount that resulted following the addition of any compound from Group I. It is conceivable that maximum growth (ca. 340 Klett units) might occur with Group II analogs if even still higher concentration of analogs were employed. This suggestion is based on the results of experiments with various concentrations of Group I analogs on the growth of staphylococci. For instance, data from Table 13 indicated that at a concentration of PSA of 250 μg/ml only half maximum growth occurred (201 Klett units) compared to the maximum growth of 400 Klett units that resulted following the addition of 2000 μg/ml or more of PSA.

Analogs from Group III failed to support staphylococcal growth in either Vitamin-free Casitone or in synthetic medium.

while the analogs in Group IV were inhibitory to even the low levels of growth able to occur in the basal media. Concentrations of Group III analogs as high as 1000 to 5000 μg/ml were unable to stimulate the growth above the control level. The complete inhibition of staphylococcal growth by the Group IV analogs, 6-AN, 5-FlN, pyrizinamide and 3-hydroxypicolinic acid, is in agreement with the findings of other workers (47, 104, 114).

The use of completely synthetic media for the growth studies showed that many niacin analogs were successfully utilized and converted into nicotinamide coenzymes by staphylococci as long as thiamine was added. For instance, addition of any of the 25 Group I analogs to a thiamine supplemented synthetic medium completely replaced the niacin requirement for staphylococcal growth (Table 7). With the addition of 3-AP or NNO to synthetic media with added thiamine, turbidity readings of 205 and 343 Klett units, respectively, were obtained. These values are comparable to that obtained with added niacin (270 Klett units). Analogs from Group III and IV also failed to stimulate staphylococcal growth in the synthetic medium, confirming the results in the Vitamin-free Casitone medium.

To insure that the niacin analogs were effective in more than the Towler strain of *S. aureus*, 10 additional strains of *S. aureus*, 3 strains of *S. epidermidis* and 3 niacin-requiring

Gram negative bacteria were tested for their ability to utilize niacin analogs in place of niacin. Our results (Table 8) showed that 3-AP (1000 μg/ml) or NDA (100 μg/ml) when added to thiamine supplemented Vitamin-free Casitone, stimulated the growth of all strains of S. aureus and S. epidermidis. Although the growth response varied somewhat from strain to strain, the growth stimulation by 3-AP and NDA was generally similar to that of niacin. Similarly, Proteus vulgaris, Proteus morgani and Shigella flexneri also utilized 3-AP and NDA as a growth factor in place of niacin. When Shigella flexneri was grown in Vitamin-free Casitone containing added 3-AP and thiamine, turbidity measurements reached 122 Klett units (24 hr) compared to 145 Klett units obtained from the addition of niacin and thiamine. Successful utilization of 3-AP, PSA and NNO as a growth factor by dysentry bacilli and by other Gram negative bacteria has been previously observed (82, 92). Thus the results of these studies strongly indicate that the utilization of niacin analogs is a general feature among the niacin requiring microbes.

The discrepancies between the negative results of previous investigators (25, 63, 68, 69) and the results of the present studies are probably due to the concentrations of analogs and the type of growth media employed in their studies. Previous workers had failed to observe any stimulatory effect on the growth of S. aureus due to the fact that very low concentrations

(10^{-5} to 10^{-4}M) of analogs had been used in their studies. These concentrations were similar to the concentration of niacin usually required for staphylococcal growth. The results reported in this dissertation (Fig. 4 and 5) indicated that the concentrations of the analogs required were at least 100- to 1000-fold higher than the 0.1 µg/ml of niacin necessary to support the maximal growth of S. aureus. As will be discussed later, the need for such high concentrations is probably due to the lack of an active transport system for the analogs in staphylococci.

The data from the present study (Fig. 4 and 5) indicated that although high concentrations of niacin or niacin analogs were tolerated by staphylococci without any adverse effects on their ability to grow, eventually a concentration was reached which caused growth inhibition. For example, addition of niacin, at concentration ranging from 0.1 to 500 µg/ml stimulated maximal staphylococcal growth and at least 0.25 µg/ml was needed for maximal stimulation of the HMP pathway. However, at a concentration of 1000 µg/ml there was a 50% reduction in growth, while glucose oxidation through the HMP pathway decreased from 21.6% to 13.4% (Table 12). When niacin was added to the medium at a concentration of 2000 µg/ml, there was complete inhibition of staphylococcal growth. The inhibitory effect of large concentration of niacin or its analogs was probably due to

the nutritional imbalance in the medium and is in agreement with the findings of other workers that concentration of niacin above 1 mg/ml inhibited growth of niacin-requiring bacteria (65).

Fig. 5 shows that there was no lag in the growth of staphylococci in the presence of the niacin analogs. This indicated that the stimulatory effects of niacin analogs on staphylococcal growth were not due to the selection of mutants capable of using the analogs. If such a selection of mutants were involved, then one would have expected to find either longer or shorter lag periods during growth on various occasions. Such growth variations in present study were not observed. This is particularly important because Das and Chatterjee (19) while investigating the effect of pyrithiamine, a thiamine analog, on S. aureus, had shown that the ability to use pyrithiamine involved the selection of mutants. Furthermore, these mutants possessed properties indicating that major biochemical changes were present in the mutant cells. Present data has conclusively demonstrated that the addition of either niacin or niacin analogs produced similar biochemical changes in the staphylococcal cells, even under non-growing conditions (Table 9 and 10). Consequently, the involvement of mutants can be eliminated from consideration.

Studies with specifically-labelled ^{14}C-glucose have demonstrated that the niacin analogs successfully replaced niacin in

stimulating the utilization of the HMP pathway in staphylococci (Table 9). The results indicated that when niacin or its analogs (Group I) were added to the growth medium, the increased amount of glucose utilized via the HMP pathway (18-24%) and the increased relative activity of the TCA cycle (4-5%), were similar in both cases. The mechanism of glucose oxidation via the HMP pathway is discussed later in this section.

The interesting finding that lower concentrations of niacin or niacin analogs were required for maximal growth than the concentrations of these substances that were required for maximal stimulation of glucose oxidation by the HMP pathway, was first discovered during studies with the analogs. Thus, the addition of 10 µg/ml of 3-pyridylcarbinol to the growth medium produced maximal growth stimulation (355 Klett units) although the cells obtained from this medium only oxidized about 10% of added glucose via the HMP pathway. A concentration of 100 µg/ml of this analog was required for both maximal growth and maximal glucose oxidation through the HMP pathway (27%). Following this discovery, the effect of lower concentrations of niacin were examined for their effects on both growth and the HMP pathway participation. It was found that addition of 0.05 µg/ml of niacin to the medium resulted in maximal growth stimulation while maximal stimulation of glucose utilization via the HMP pathway required a concentration of at least 0.25 µg/ml (Table 12). The absolute difference between the concentrations of

niacin allowing maximal growth stimulation and maximal stimulation of the HMP pathway was only o.2 μg/ml and had not been observed prior to the studies with the analogs. With the analogs such as PSA or 3-pyridylcarbinol this difference was much more readily apparent. For example, with 3-pyridylcarbinol the difference was 90 μg/ml and for PSA it was 1750 μg/ml. These findings clearly proved that the maximal operation of the HMP pathway was not a necessary requirement for the maximal growth of staphylococci.

The minimal contribution of glucose oxidation through the HMP pathway under conditions that will still allow staphylococci to grow at their maximal rate is apparently less than 1%. Although the usual percentage of glucose oxidized by staphylococci via the HMP pathway is 7 to 8% in Vitamin-free Casitone growth medium not supplemented with niacin, this value was reduced to 0.3% when 6-AN was included along with niacin in the growth medium (Table 14). Streightoff (114) had previously shown that the growth inhibitory effect of 6-AN was nullified by the inclusion of niacin in the growth medium. However, he had not studied any biochemical properties of these cells. In the present study when 100 μg/ml of 6-AN was added to a medium containing 0.05 μg/ml of niacin, the staphylococci were able to grow at their normal rate. However, when these cells were used to estimate the glucose catabolic pathways, only 0.3% of the

glucose was found to be oxidized via the HMP pathway, the lowest value ever observed with staphylococci. Apparently this small fraction is able to supply the pentoses and NADPH necessary for growth. With the use of isotopic studies, it has been shown that in E. coli, about 1% of glucose carbon passing through the oxidative part of the HMP pathway is converted into phosphoribosyl pyrophosphate, an intermediate for nucleotide synthesis (11, 55). On the basis of these findings it appears that one of the primary functions of HMP pathway is to generate NADPH for reductive biosynthesis.

From these data several conclusions can be drawn:

1. Niacin analogs can completely replace niacin for growth and for stimulation of glucose utilization via the HMP pathway.

2. High levels of operation of the HMP pathway (i.e., 20-30%) are not required for maximal growth rate. When as little as 1% of the glucose is being catabolized via the HMP pathway, that rate is sufficient to allow staphylococci to grow at their maximal rate under the conditions studied.

3. The addition of niacin or its analogs can stimulate the utilization of the HMP pathway and the TCA cycle activity (when thiamine is present) in the complete absence of growth.

Impaired function of the HMP pathway in S. aureus due to the addition of 6-AN under growing and resting cell conditions, was observed in our study (Table 14). Such an inhibition of the HMP pathway has already been demonstrated following 6-AN administration in mammalian system (22,40,41,64). The toxic effects in animals, resulting from the administration of 6-AN have been shown due to the formation of NAD(P) analogs in vivo, which are inhibitory to a number of NADP-dependent dehydrogenases (17,41,64). Herken (41) observed that there was a considerable accumulation of 6-phosphogluconate compared to glucose-6-phosphate, in the brains of 6-AN treated animals (0.2 and 2 μmoles of G6P and 6PG/g dry weight, respectively) indicating a significant reduction in 6PGD activity. Accumulation of these substrates was not observed in the control animals which did not receive 6-AN.

In parallel studies in this laboratory, it was shown that the addition of 6-AN to the growth media resulted in a similar accumulation of glucose-6-phosphate and 6-phosphogluconate (1.3 and 7.3 μmoles of G6P and 6PG/g, respectively) in staphylococci (46). This 5-fold increase of 6PG over G6P strongly suggested that the site of action of 6-AN in S. aureus was also at the 6PGD level. In the present study the activities of G6PD and 6PGD from 6-AN grown cells were not tested. However, the resuts of the isotope experiments with intact cells demonstrated

that $^{14}CO_2$ evolution from glucose-1-^{14}C was substantially reduced, indicating that the two initial enzymes (G6PD and 6PGD) of the HMP pathway were inhibited. On the basis of these findings, it is not possible to conclude that reduction in the HMP pathway utilization, accompanied by a 10-fold increase in the substrates in S. aureus, was due to the formation of coenzyme analog of NAD(P). However, it is clear from the present study that in S. aureus the site of action of 6-AN is also at 6PGD level, as observed in mammalian system (41).

The results of the analog study indicated that none of the hydroxylated pyridine derivatives replaced niacin, either for growth or for the operation of the glycolytic pathways. Data from Table 15 suggested that 6-hydroxynicotinic acid (6-HN) stimulated the TCA cycle activity under certain conditions and this thiamine-like effect was observed only in the cells that had been previously grown in the presence of 6-HN and subsequently incubated in a medium containing added niacin. The mechanism for this stimulation of the TCA cycle activity by 6-HN is not presently understood. One possibility is that the analog may cause derepression of thiamine biosynthesis in staphylococci so that the cells may have enough of this vitamin for the active operation of the TCA cycle. Derepression of thiamine synthesis in E. coli and Enterobacter species by compounds such as adenine and phenylalanine, has been demonstrated (58,83).

Ellinger et al. (25) suggested that the growth promoting activity of some pyridine derivatives for various niacin requiring bacteria was due to slight contamination with niacin or niacinamide of the pyridine derivatives. The compounds which stimulated growth fully, were also considered to be heavily contaminated. These suggestions were not based on any direct experimental evidence. However, since 3-acetylpyridine (3-AP) and other analogs were required in such large amounts (1000 μg/ml) for staphylococcal growth stimulation, the possibility was considered that this growth stimulation might actually be due to contamination of the 3-AP by niacin or niacinamide. Since 3-AP is a volatile liquid, while niacin and niacinamide are not volatile, large quantities of this compound were spotted on paper chromatograms. Using different solvent systems, UV spectra and chemical reactions, no contamination of 3-AP by niacin or niacinamide was detected. With this technique it would be possible to detect as little as 0.05 μg of niacin in the 1000 μg of 3-AP. Thus, when 5000 μg of 3-AP containing 0.05 μg of niacin was spotted on paper, this low amount of niacin was detected in the mixture. On the basis of these findings and the limitation of the technique, any contaminating amount of niacin must be present in a concentration less than 0.05 μg in 1000 μg of 3-AP. Such a low level of niacin, even if it was present, could not account for the results reported in the present study

with the analogs. Using similar techniques it was also observed that the growth stimulatory effects of NDA and quinolinic acid were not due to their contamination with niacin and/or niacinamide.

The possibility was considered that the stimulatory effect of the analogs on growth, glycolysis and on nicotinamide coenzymes was due to the induction or derepression of niacin biosynthesis. In Saccharomyces cerevisiae the addition of the thiamine analog oxythiamine (15 μg/ml) to the growth medium stimulated the biosynthesis of thiamine (107, 108). In the present studies, with the use of ^{14}C-quinolinic acid and ^{3}H pyridine-N oxide, it was shown that the radioactivity from the analogs was directly incorporated into the nicotinamide coenzymes (46). If the induction of niacin synthesis in the staphylococcal cells was due to the addition of niacin analogs, then the incorporation of radioactivity from the analogs into NAD(P) would not have occurred. On the basis of these results it was concluded that the stimulation of NAD(P) synthesis as a result of the addition of niacin analogs was not due to the induction or the derepression of niacin biosynthesis in staphylococci. The precise mechanism of this formation of nicotinamide coenzymes from the analogs is not presently understood. From the isotopic data, and from various other types of evidence, it was concluded that the stimulation of growth, of glycolysis and of NAD and

NADP synthesis in staphylococci were all due to the increased biosynthesis of unaltered nicotinamide coenzymes from the analogs after the analogs had been modified biochemically within the cells (46).

The need for high concentration of analogs to bring about changes in the growth and in biochemical characteristics was thought to be due to the reduced permeability for these compounds.

Considerable evidence was obtained that the uptake of ^{14}C-niacin by staphylococci proceeded by a physiological mechanism involving active transport (Fig. 6 to 10). The results of the uptake studies indicated that niacin was taken up and retained against a concentration difference and that the transport appeared to be temperature-dependent (Fig. 8) and pH sensitive (Fig. 7). It should be noted that the niacin is rapidly converted to NAD and NADP within the cells (33, 46). Data presented in Fig. 10 showed that the rate of niacin uptake was increased when there were higher niacin concentrations in the medium and that this process was saturated at a concentration of 10^{-7}M (Fig. 10). Similarly, the maximal rate of niacin uptake was found to occur at pH 5.5 and at 50 C. There was relatively no uptake either at 4 C or at pH 4.0 and 8.0 (Fig. 7 and 8), indicating the transport system was both pH and temperature dependent.

The uptake of ^{14}C-quinolinic acid and ^{14}C-isoniazid by niacin-deficient staphylococcal cells was negligible when the suspensions were incubated with 1, 2, 10 or 50 μg/ml of the analogs, for 2 hr at 37 C, in the presence of 1% glucose. Only a 2% increase in the uptake was observed when the concentrations of either isoniazid or quinolinic acid were increased to 500 μg/ml. Although the ^{14}C of the carbonyl-labeled does not end up in NAD and NADP, the quinolinic acid-6-^{14}C does (46). Although this uptake was low when compared to that of niacin, it was sufficient to induce the biochemical changes in the staphylococcal cells reported earlier (Table 18). Similarly, the uptake process for the analogs did not appear to be influenced by changes in pH or in temperatures. On the basis of these results, it appeared that the niacin analogs entered the staphylococci by simple passive diffusion. An analogous situation was noted by Kawasaki et al. (57), who found that the uptake of thiamine in *E. coli* occurred by an active transport process. Upon isolation of a mutant of *E. coli* that was defective in thiamine uptake, they were able to demonstrate that the thiamine concentration required for the half maximal growth for this mutant strain was approximately 0.037 μg/ml, which was a concentration about 150-fold higher than that required for the parent strain. Results with staphylococci in the present study also indicated that 100- to 1000-fold higher concentrations of niacin analogs than

niacin were required for maximal growth, due to the lack of an active transport for the analogs.

E. coli, *S. aureus* and *B. megaterium* (resistant to isoniazid) have been shown to utilize extremely small amounts of isoniazid compared to sensitive strains of *Mycobacterium tuberculosis* (128). In the present study it was also found that staphylococci took up extremely small amounts of isoniazid, even after exposure of cells for 6 hr (Fig. 11). On the basis of the uptake studies with the analogs (Fig. 11 and 12), it is strongly suggested that the need for high concentration for growth stimulation and for the biochemical changes, is due to the absence of an active transport system for the analogs in *S. aureus*. The analogs probably enter the cells by simple passive diffusion after which they are able to cause changes similar to those caused by niacin.

V. SUMMARY

S. aureus catabolized glucose by both the Embden-Meyerhof (EM) and the hexosemonophosphate (HMP) pathways, as determined with ^{14}C-labeled glucose. The relative activities of these pathways, and of the TCA cycle, were influenced by the inclusion of niacin and/or thiamine in the growth medium. When > 0.25 μg/ml of niacin was added to 2% Vitamin-free Casitone, the proportion of glucose metabolized via the HMP pathway by S. aurues was stimulated 3-fold. When 0.1 μg/ml of niacin was added, the growth was maximally stimulated but increased NAD(P) synthesis or HMP pathway activity was not observed. Under growing conditions, increased glucose oxidation via the HMP pathway and TCA cycle activity were correlated with the increased synthesis of NADP-specific glucose-6-P dehydrogenase and isocitrate dehydrogenase, respectively. When the vitamin supplementation was made to resting niacin-deficient cells, metabolic changes similar to those in growing cells occurred, even in the presence of protein inhibitors such as chloramphenicol, puromycin or actinomycin D. Since the specific activities of the enzymes tested remained unchanged under conditions that precluded new enzyme synthesis, the enzyme levels were not the limiting factors for glucose oxidation via

the HMP pathway.

In order to determine if either NAD and/or NADP were regulating the HMP pathway activity, the staphylococcal suspensions were starved for 6 hr in buffered-glucose (pH 7.0), under conditions that allowed the NAD to decrease and NADP to remain constant. During the period of starvation the initial NAD level decreased to 1 μmole/g and the percent of glucose oxidized via the HMP pathway decreased to 9%, reaching a value of 6% after 6 hr at which time the NAD content was further reduced to 0.5 μmole/g. When niacin was added, the NAD levels and the HMP activity in these cells were increased to 1.2 μmoles and 16%, respectively, after 2 hr of additional incubation. These results indicate that NAD is regulating the activity of this expandable (6 to 22%) HMP pathway, by increasing NADP turnover via the transhydrogenase system.

Any of several 1-, 2-, 3- and 4-substituted niacin analogs completely replaced the niacin requirement for both growth and the HMP pathway utilization when added at high concentration (ca. 1000 μg/ml) to either a synthetic medium or Vitamin-free Casitone supplemented with thiamine. On the basis of their effects on growth and on the HMP pathway the analogs were classified into 4 groups. The 22 group I analogs, including pyridine-N-oxide, pyridine-3-sulfonic acid and 3-acetylpyridine, stimulated both growth and the HMP pathway maximally while the 9 group II analogs, such as picolinic acid, stimulated growth

and the HMP pathway to a lesser extent. There were 13 analogs from group III, including 6-hydroxynicotinic acid, that were without effect while 4 analogs from group IV, including 6-aminonicotinamide, were inhibitory. Parallel experiments in this laboratory using labeled quinolinic acid, isoniazid and pyridine-N-oxide indicated that the analogs were converted to NAD(P) and not to their analogs. On the basis of the permeability studies with the labeled compounds and ^{14}C-niacin, it was suggested that the need for high concentrations of analogs for growth was due to the absence of active transport for these compounds and the analogs entered the cells by simple passive diffusion to cause biochemical changes similar to those caused by niacin.

VI. LITERATURE CITED

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APPROVAL SHEET

The dissertation submitted by Manohar Wadke has been read and approved by the undersigned faculty members.

The final copies have been examined by members of the Dissertation Committee and the signatures which appear below verify the fact that any necessary changes have been incorporated and that the dissertation is now given final approval with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Dr. H.J. Blumenthal, Advisor — 3/17/71 Date

Dr. J. Goldberg

Dr. T. Hashimoto

Dr. G. Plummer

Dr. W.W. Yotis